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(54) Title: ASSAY TO DETECT SUBSTANCES USEFUL FOR THERAPY

(57) Abstract: The present invention relates to the use of effectors/regulators for Rab and Rho GTPases in *in vitro* and *in vivo* assays that recapitulate and measure the role of these effectors/regulators in membrane transport and membrane-cytoskeleton interactions in the endocytic pathway as novel targets to find therapeutic drugs to prevent or inhibit cancer cell growth and arrest cancer cell inva-  
siveness as well as for stimulating and/or restoring endocytic transport and phagosome maturation in cells infected by intracellular parasites, which drugs are therefore useful for the therapy and optionally also the prophylaxis of cancer and infectious diseases. In addition, the present invention is also directed to kits useful as a means to detect drugs suitable as anti-cancer and anti-infectious diseases drugs.



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### Assay to Detect Substances Useful for the Therapy

The present invention relates to the use of effectors/regulators for Rab GTPases in *in vitro* and *in vivo* assays that recapitulate and measure the role of these effectors/regulators in membrane transport and membrane-cytoskeleton interactions in the endocytic pathway as novel targets to find therapeutic drugs to prevent or inhibit cancer cell growth and arrest cancer cell invasiveness as well as for stimulating and/or restoring endocytic transport and phagosome maturation in cells infected by intracellular parasites, which drugs are therefore useful for the therapy and optionally also the prophylaxis of 1) cancer and other proliferative (skin repair diseases such as psoriasis), invasive or cell migration disorders (endometriosis, atherosclerosis, inflammation and allergic diseases), 2) infectious (bacterial and viral) diseases, 3) diabetes, 4 ) Alzheimer's disease. In addition, the present invention is also directed to kits useful as a means to detect drugs suitable as anti-cancer and anti-infectious diseases drugs.

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A class of molecules shown to play an important role in the regulation of intracellular transport and organelle function is represented by Rab proteins, small GTPases of the Ras superfamily, which are required in virtually every transport step which has been investigated. Similar to other GTPases, these molecules use the conformational change induced by GTP hydrolysis to regulate downstream events necessary for vesicle formation, docking and fusion. In the GTP-bound, active form Rab proteins bind to effector proteins and in this way transmit their signal to the transport machinery. For example, in membrane docking and fusion, Rab proteins regulate the activity of SNAREs. SNAREs are integral membrane proteins that by pairing on opposite membranes engaged in docking lead to membrane fusion. The pairing of SNAREs requires the activity of Rab proteins and therefore of Rab effectors. For example, endosome membrane docking requires the presence of the Rab5 effector EEA1 which, upon bridging the two opposite membranes, allows SNAREs to pair in trans, thus leading to membrane fusion (Chistoforidis et al., 1999a). The role of Rab proteins is not restricted to membrane docking and fusion but recent data provide evidence that Rab proteins also regulate the association with and motility of vesicles along cytoskeletal filaments. Membrane-cytoskeleton interactions play an important role in

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determining the intracellular distribution and motility of organelles and transport vesicles. The cytoskeleton is made of different types of filaments, actin filaments and microtubules being the best characterised. Actin filaments and microtubules undergo continuous remodelling to serve the needs of the cell in cell architecture, motility, and organelle movement. Microtubules play a pivotal role in cell division but also constitute tracks along which cargo is transported across long distances such as between the periphery and the centre of the cell, from the basal to the apical pole in epithelial cells, or along the axon in neurons. Actin filaments are well known to function in muscle contraction. They are also important for cell motility, cell shape and vesicular transport. Different proteins are necessary to attach the organelles and transport vesicles to, and move them along, these filaments. Rab5 has recently been demonstrated to regulate the attachment of endosomes to, and motility along, microtubules. This suggests that Rab5, and in general members of the Rab proteins family, regulate various aspects of intracellular transport, including the ability of organelles to move along cytoskeletal tracks.

The identification of a large number of effectors and regulators for the small GTPase Rab5 provides a molecular explanation for the multiplicity of functions of Rab5 and allow to predict similar mechanisms for other Rab GTPases. Rab5 regulates a molecular network of several effector proteins, each contributing a specific function in membrane organisation, vesicle formation, vesicle and organelle movement, membrane docking and fusion. Most importantly, by functioning in a cooperative fashion Rab5 effectors modify the membrane environment and thus contribute to the biogenesis of the early endosome membrane. This mode of action is exemplified by the following mechanism elucidated by the present inventors. Upon activation by the effector/exchange factor Rabaptin-5/Rabex-5 complex, Rab5 locally recruits and activates phosphoinositide PI3-Kinases, leading to the generation of PI(3)P and consequently allowing the membrane recruitment of other Rab5 effectors (e.g. EEA1; see below) that bind to both Rab5:GTP and PI(3)P (Christoforidis et al., 1999b). Furthermore, Rab5 effectors are engaged in the formation of oligomeric complexes on the early endosome membrane (McBride et al., 1999). By the same criteria, other Rab proteins present in the early endosomes would be expected to recruit multiple effectors within a separate membrane environment. Consistent with this, studies using (green fluorescent protein) GFP-tagged Rab5, Rab4, and Rab11 have demonstrated that these GTPases are present in separate sub-compartments of the early endosome membrane. Endosomes are therefore organised as a mosaic of different Rab-domains created through the recruitment of specific effector proteins, which co-operatively act to generate a topologically restricted and functionally specialized environment on the endosome membrane.

Laying at the core of the organelle biogenesis and membrane transport mechanisms, Rab GTPases and their effectors are placed in a strategic position to regulate and mediate the trafficking of cellular membrane and soluble components along the biosynthetic and endocytic pathways. Their function includes the regulation of transport of cellular constituents (proteins and lipids) that are implicated in pathological alterations. Consequently, the present inventors' understanding of the mechanisms underlying membrane trafficking and membrane-cytoskeleton interactions, has applications in biotechnological research. Applications are in particular possible for those diseases where modulation of the membrane trafficking properties of endosomes provides an opportunity for therapeutic intervention. The main research areas for which this knowledge has proven to have important implications are: 1) cancer and other proliferative (skin repair diseases such as psoriasis), invasive or cell migration disorders (endometriosis, atherosclerosis, inflammation and allergic diseases), 2) infectious (bacterial and viral) diseases, 3) diabetes, 4 ) Alzheimer's disease. In the following section, the Rab GTPases and Rab effectors machinery will be presented in the context of the development of a therapeutic strategy for the treatment of these diseases.

### 1. Cancer

The molecular analysis of the transport machinery represented by Rab effectors operating in the endocytic pathway is relevant to two related aspects of the molecular mechanisms underlying tumor growth: intracellular signalling and cell spreading.

#### *Intracellular signalling*

Cell growth is regulated by growth factors that by binding specific receptors on the cell surface trigger a signalling cascade that culminates with the regulation of gene expression. Growth factors and their receptors are important mitogens both in normal and in transformed cells. Mutations in their genes or overexpression can cause tumors or stimulate tumor cell growth. For example, many tumors overexpress growth factors and surface tyrosine kinase receptors thus enhancing tumor cell growth. Growth factors and growth factor receptors have therefore been considered as excellent targets for new therapeutic drugs in the treatment of cancer and other disorders of excessive cellular proliferation. For example, the Herc2 receptor implicated in metastatic breast cancer provided the target for the development of Herceptin, a monoclonal antibody therapeutic from Genentech. The strategies pursued so far include the search for compounds that i) block ligand-receptor interactions, ii) the kinase activity of the receptors or the subsequent interactions with downstream factors of the signalling cascade. Another approach

consists of the search for drugs that modulate receptor trafficking through the endosomal system and result in receptor downregulation at the plasma membrane. Growth factor receptors and their bound ligands are internalised into endosomes, and endocytic trafficking of these molecules plays a critical role not only in attenuating the signalling response but also in establishing and controlling specific signalling pathways. The ability of growth factors to trigger signaling depends on the surface appearance of their receptors. This is determined by the kinetics and extent of receptor 1) endocytosis, 2) recycling to the cell surface and 3) transport to, and degradation in, late endocytic compartments (Sorkin et al., 1991). Molecules that can modulate these events and decrease the fraction of growth factor receptors on the plasma membrane are therefore valuable tools for an anti-cancer therapy. For example, enhanced degradation of epidermal growth factor receptor correlates with the lack of epidermal growth factor- induced proliferation and mitogen-activated protein kinase stimulation (Caraglia et al., 1999). Given their established role in the regulation of endosome structure and function, Rab GTPases and their network of Rab effectors represent an ideal target for screening of molecules that can reduce the surface content of growth factor receptors and, consequently, inhibit the growth properties of tumor cells. Rab5 for example regulates the internalisation of receptors from the plasma membrane into clathrin-coated vesicles (CCV) as well as the subsequent delivery of these carriers to early endosomes. Other Rab proteins such as Rab4 and Rab11 regulate the sorting function of early and recycling endosomes and, consequently, the recycling to the surface of growth factor receptors. The small GTPase Rab7 regulates transport of molecules from early to late endosomes and lysosomes, the degradative endocytic organelles.

The impact of Rab GTPases and their effectors/regulators on mitogenic signaling also includes the trafficking of signaling molecules downstream of growth factor receptors. An increasing number of components of the signalling machinery have recently been localised to early endocytic organelles. This remarkably specific localisation is very likely an important element in the signalling process. Such localisation depends on the structural and functional properties of the early endosomes, which in turn depends on the activity of Rab GTPases and their corresponding effectors/regulators. For example, the localisation of FYVE finger proteins depends on the synthesis of PI(3)P on the early endosome membrane (Christoforidis et al., 1999b). In the absence of this phosphoinositide these proteins are no longer capable of residing on early endosomes and are therefore released into the cytosol. Several novel signaling molecules possess a FYVE finger and, consequently, their ability to reside on early endosomes and to participate in the signaling cascade depends on the synthesis of PI(3)P. This concept is

corroborated by recent data indicating that hVPS34, the PI3-K that produces PI(3)P is required for mitogenic signaling (Siddhanta et al., 1998). The synthesis of PI(3)P is subjected to the regulation by Rab5, since PI-3 kinases (including hVPS34) are Rab5 effectors (Christoforidis et al., 1999b). Indeed, recent studies of the present inventors have demonstrated that the production of PI(3)P is stimulated by Rab5 and inhibited upon removal of Rab5 from endosomes. These observations therefore establish a direct link between the activity of Rab5 and its effectors such as PI-3 kinases and the membrane recruitment of signalling molecules to early endosomes and their ability to function in the signalling process.

#### *Cell spreading*

The growth of human tumors depends not only on the proliferative advantage characteristic of cancer cells but also on the ability of these to invade tissues and to colonise different organs. The ability of tumors to form metastatic tumours depends on the invasive properties of transformed cells. In addition, cancer cells require a higher supply of nutrients and, therefore, require de novo formation of blood vessels. Clearly, the spreading of endothelial cells during angiogenesis that occurs concomitantly with tumor growth also requires an increase in cell motility. Cell locomotion is therefore a central mechanism in tumor cell invasiveness and metastasis. On the one hand, cell spreading and invasiveness depend on the signalling function of various growth factor and growth factor receptors. In many different cell types, including tumor cells, EGFR signaling produces a pleiotropic response that includes mitogenesis or apoptosis, enhanced cell motility, protein secretion, and differentiation or dedifferentiation. The formation of new blood vessels is instead tightly regulated by specific growth factors that target receptor tyrosine kinases (RTKs) such as vascular endothelial growth factor (VEGF) (McMahon, 2000). A novel approach to treat human cancers emerging from these studies consists in the development of therapeutic strategies aimed at blocking tumor cell invasiveness and deregulated angiogenesis through the inhibition of various growth factor receptor signaling pathways (both in tumor cells and in the neighbouring tissue such as in endothelial cells). The potential use of Rab GTPases and their effectors/regulators as targets for the screening of molecules that can reduce the signaling function of growth factor receptors applies therefore not only for the mitogenic signaling but also for the spreading of tumor cells. On the other hand, the use of Rab GTPases and their effectors/regulators can also be extended to the cellular machinery that executes the signaling program resulting in increased cell locomotion. Cell locomotion occurs by a complex mechanism involving the coordinated activity of both the cytoskeleton - actin and microtubules - and membrane trafficking - especially endocytic and recycling structures. The structural/functional

properties of endosomes are important elements of the cell motility apparatus. For example, the endocytic cycle between endosomes and the plasma membrane sustains the deposition of adhesion molecules to the leading edge of motile cells. Consequently, the function of molecules that play an important role in endocytic transport can be rate-limiting important factors for the survival and spreading of cancer cells. This proposal is supported by recent studies that demonstrate that the small GTPase Rab5 is required for cell motility. The use of Rab GTPases and their effectors/regulators is therefore here applied for the screening of molecules that can reduce the ability of the cell to respond to mitogens through increased cell motility and, consequently, inhibit the spreading of tumor cells.

The described application of Rab effector intervention as a means of inhibiting or regulating cell motility defines the potential of the intervention for the treatment of other diseases that depend on cell locomotion such as endometriosis, atherosclerosis and monocyte migration into plaques, asthma and allergic diseases, and any other disease dependent on cell migration or locomotion.

## 2. Infectious diseases

Another area according to the present invention where membrane trafficking molecules, i.e., Rab GTPases and their effectors/regulators, can serve as therapeutic targets is that of infectious diseases. Cells internalise soluble ligands through endocytosis and large particles through phagocytosis. The latter mechanism is exploited by parasites that in this way gain access to the endomembrane system of the host cell. Increasing numbers of studies indicate that phagocytosis is regulated by the same molecular principles that govern endocytosis and recycling. For example, amphiphysin II and dynamin 2, which participate in receptor-mediated endocytosis, are also required for phagocytosis at the stage of membrane extension around the bound particles. Since the phagocytic process results in the internalisation of a large portion of the plasma membrane, mechanisms exist to ensure rapid renewal of plasma membrane. Given the role of Rab proteins in the regulation of membrane transport, it is obvious that they play an important role in the phagocytic cycle as well. Indeed, recent studies have shown that Rab11 operates in an endocytic compartment which is essential for phagocytosis. The function of endocytic Rab GTPases and their corresponding effectors/regulators are therefore expected to play an important regulatory role in the host cell invasion by parasites mediated by phagocytosis (Rab5) and in the compensatory membrane recycling (Rab4, Rab11) sustaining this process.

Once internalised, engulfed parasites or particles reside intracellularly in phagosomes. Under

normal conditions, phagosomes mature by docking and fusing progressively with early endosomes, late endosomes and lysosomes, thus causing the destruction of their content due to the low pH and increasing exposure to hydrolytic enzymes. However, it is well established that certain microbes can sabotage the cellular defence mechanisms and survive intracellularly. This is the case for example for *Mycobacterium tuberculosis* that once internalised by phagocytosis resides within the phagosome without coming in contact with harmful organelles such as lysosomes. A possible strategy to facilitate the killing of the pathogen is to re-activate the membrane trafficking route, thus resuming the maturation of the phagosome and, therefore, the exposure of the internalised bacterium to low pH and harmful hydrolytic enzymes. The strategy should focus on different Rab proteins each regulating a distinct stage of phagosome maturation (i.e. Rab5, Rab4, Rab11, Rab7).

Another area where Rab GTPases and their effectors can be used for therapeutic intervention is that of viral infection, especially HIV. AIDS continues to be cause of many deaths both in developed and under-developed countries. It has been recently established that, besides CD4, members of the family of chemokine receptors are required as co-receptors, which in conjunction with CD4, allow the virus to enter cells. Downregulation of chemokine receptors by endocytosis and inhibition of receptor recycling protects cells from HIV. These observations imply that receptor internalization and inhibition of receptor recycling represent novel therapeutic strategies to prevent HIV infection and therefore combat AIDS. Since Rab GTPases and their effectors/regulators regulate the trafficking of receptors to the cell surface, these molecules represent new targets for therapeutic agents aimed at decreasing the surface expression of HIV receptors and therefore inhibit HIV entry into cells and prevent HIV infection.

### 25 3. Diabetes

Another area where the endocytic trafficking apparatus has potential commercial applications is the trafficking of glucose transporters and the defect in this process, which is a cause of diabetes. In fat and muscle cells, stimulation by insulin causes the relocation of glucose transporters (e.g. GLUT4) from intracellular stores to the cell surface. These intracellular stores are thought to be specialised membrane domains of the recycling endomembrane system. Insulin sensitivity depends on the surface levels of such glucose transporters (GLUTs). Under physiological conditions, the increase in muscle insulin sensitivity of glucose transport observed after exercise is due to translocation of more GLUT-4 to the cell surface. Under pathological conditions, GLUTs trafficking is perturbed. Certain forms of diabetes are in fact caused by deficiency in the



surface appearance of the GLUTs. This implies that the elucidation of the transport machinery that regulates the trafficking of membrane proteins, including GLUTs, along the endocytic and recycling pathway offers therefore interesting opportunities for intervention in diabetes. In particular, the discovery of Rab effectors that play a role in the transport to endosomes and from  
5 endosomes to other destinations, e.g., the cell surface or to degradative compartments, identifies potential targets for the screening of drugs that, by altering the trafficking properties of GLUTs, can restore or stimulate their delivery to the plasma membrane in response to insulin.

#### 4. Alzheimer's Disease

10 At the core of the molecular mechanism underlying the generation of Alzheimer's disease lies the aberrant processing of the Amyloid Precursor Protein (APP) by  $\beta$ -secretase to form  $A\beta(1-42)$ . It is at present unclear where precisely the aberrant cleavage occurs, but endosomes have been implicated as the compartment or one of the compartments where processing of APP occurs. This implies that 1) APP is transported to endosomes and follows the endocytic/recycling  
15 pathway and 2)  $\beta$ -secretase activity is localised to endosomes. Since Rab GTPases and their effectors/regulators regulate the trafficking of molecules (lipids and proteins) in the endocytic pathway, these molecules represent new targets for therapeutic agents aimed at decreasing the presence of APP in endocytic compartments and/or the presence of  $\beta$ -secretase activity in endocytic compartments, therefore inhibiting the generation of beta-amyloid and retarding and or  
20 preventing the onset/progression of Alzheimer's disease.

Based on the above experimental results, the object of the present invention was to develop an assay useful for the detection and identification of molecules (pharmaceutical drugs). In order to achieve this object, the present inventors developed the strategy that molecules (proteins)  
25 interacting with GTPases such as GTPases of the Rab family and, thus, functioning at the level of endosome trafficking and coordinating the interaction of these organelles with the cytoskeleton, can provide novel targets for molecules (pharmaceutical drugs) aimed at inhibiting cell locomotion and invasiveness (and thereby cancer) as well as infectious (both viral such as AIDS and bacterial and also eukaryotic), allergic, and inflammatory diseases, diabetes, Alzheimer's  
30 disease, endometriosis, and atherosclerosis. A further object of the present invention was to provide a kit comprising molecules interacting with GTPases (effector or regulator proteins/molecules; for exact definitions see further below), which kit is useful as a means for the detection and identification of pharmaceutical drugs exhibiting properties making them preferred

drugs for treating cancer, inflammatory, allergic, and infectious diseases (eukaryotic, prokaryotic and viral) including AIDS but also atherosclerosis, endometriosis, diabetes and Alzheimer's disease. Hereinafter, these diseases are termed "the above mentioned diseases".

5 The following definitions are given in order to more fully specify the terms as used in the present application. "**Invasiveness**" refers to the migration and spreading of cancer cells or other cells such as endothelial cells (rather than the invasion of bacteria). "**GTPase-interacting protein**" or "**regulator/regulatory protein/regulatory molecule**" refers to any protein that interacts with the GTPase under any nucleotide conformation and for any purpose. GTPase-interacting  
10 proteins/regulators regulate the nucleotide-bound state of the GTPase, i.e., the GTPase activating protein (GAP) interacts with the GTP-bound GTPase and down regulates the activity of the GTPase by stimulating the hydrolysis of GTP. A GDP/GTP exchange factor promotes nucleotide exchange, thereby activating the GTPase. "**Effector**" or "**effector/effector protein/effector molecule**" is defined to mean a protein or a protein complex that interacts with the GTPase only  
15 if the GTPase is in the GTP-bound, also called active, form, that by binding stabilises this conformation and that mediates the "effect" of that GTPase. Effectors transmit the function of the GTPase, Rab is upstream and recruits the effectors which do the work. Regulators instead switch the Rab on and off. Rab-on (=GTP) binds the effectors and is functional. Thus, both effectors and regulators may therefore also be termed GTPase effectors and GTPase regulators,  
20 respectively. Additionally, it is apparent for the skilled reader that the term "**regulator**" is broader than the term "**effector**" and includes the latter.

The above mentioned strategy implies the first step of employing known effectors/regulators or identifying novel effectors/regulators of Rab or of other (small) GTPases, specifically regulating  
25 endosome trafficking and endosome-cytoskeleton interactions, and the additional step of designing an *in vitro* and/or *in vivo* assay to screen for inhibitors or activators of these effector/regulator molecules. In particular, the strategy includes the identification of substances useful as pharmaceutical drugs due to their interference with the process of cell locomotion, i.e., it includes the identification of inhibitors of membrane-cytoskeleton interactions.

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The above object in mind, the present inventors established an assay system based on the use of such effectors/regulators, as defined hereinbefore, as targets for pharmaceutical drugs useful in the therapy of cancer and infectious diseases and any other disease which affects or requires the function of the endocytic/recycling trafficking machinery. According to a preferred embodiment

of this aspect of the present invention, an assay is provided using both an effector/regulator and a Rab protein (or any other small GTPase). Accordingly, one aspect of the present invention is the use of the above mentioned GTPase effector/regulator molecules or proteins as targets in an *in vitro* or *in vivo* assay system to detect substances useful as a pharmaceutical/therapeutic agent for the prophylaxis and the treatment of the above mentioned diseases (including cancer, allergic, inflammatory, and infectious diseases, diabetes, endometriosis, atherosclerosis and Alzheimer's disease). Another object of the present invention is a kit useful for carrying out the assay of the invention. The kit necessarily contains at least one type of effector/regulator molecule. At least one GTPase effector/regulator molecule is either native and biochemically purified from a vertebrate, or recombinant and biochemically purified from bacterial cultures, from yeast cultures, or from other cultured eukaryotic cells, in either case labelled by a covalent modification or radioactivity suitable for use in the assay.

According to a preferred embodiment of this aspect of the present invention the kit additionally contains at least one type of GTPase and/or endosomal membrane fractions obtained by subcellular fractionation and labelled by internalisation of transferrin, either fluorescently labelled or labelled by any other modification that allows its detection, and/or cytosolic extracts, and/or an ATP-regenerating system and/or a number of chemicals to be tested for their suitability as a drug against any of the above mentioned diseases (cancer, endometriosis, diabetes, atherosclerosis, allergic, infectious, inflammatory diseases). Other specific and preferred embodiments of both the kit and the use according to the present invention may be readily taken from the claims attached hereto.

Drugs exhibiting any of the above activities include those molecules (including peptides) mimicking the peptide structure of the respective target molecule. Typical representatives of the above classes are molecules/substances that carry one or more of the following functional groups: a halide atom bound to an alkyl, alkenyl, alkynyl, or aryl residue, an alcohol group (primary, secondary, tertiary), an ether group, a carbonyl function (aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group including a lactone group, a benzyl, phenyl, tolyl, tosyl, sulfonyl group, an amino group (primary, secondary, tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group, and a quinone group. Other representatives of suitable drugs are organometallic compounds, sterol moiety(ies)-containing molecules,  $\beta$ -hydroxy carboxylic acids, inorganic acids, and complexes such as metallocenes; nucleic acids,

cytokines, hormones, antibodies, or oligopeptides comprising up to 20, preferably 8, 10, or 12, amino acid residues.

Preferably, the antibodies are polyclonal or monoclonal antibodies, or fragments thereof, humanised or even human antibodies (obtained for example by a phage display method), inhibitory or stimulatory, raised against and targeted towards any of the aforementioned GTPase effectors. A preferred nucleic acid is genomic DNA, cDNA, or mRNA, or a fragment thereof, an oligonucleotide, an oligoribonucleotide, all being based on or derived from any of the GTPase effector gene sequences having any of the sequences as depicted in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, and 15, or gene therapy vectors derived from the aforementioned GTPase effector gene sequences.

The advantage (and the gist) of the present invention consists in (1) the development of a comprehensive screening method based on molecular networks, (2) the sensitivity of the assays, and (3) the selectivity of the targets. The activity of the factors under control of the central switch can be responsible for coordinating various interconnected functions such as (a) endocytosis, (b) the distribution, i.e., sorting of proteins and lipids within endosomes and from endosomes to other intracellular locations (e.g. interactions of phagosomes with endosomes and lysosomes), (c) the motility of endosomes along the cytoskeleton, and (d) cell migration. This multiplicity of factors responsible for a wide number of cellular functions is defined as a *molecular network*. The present invention resides in enabling the skilled person to study the molecular network of many different GTPases, in particular those GTPases localised to endosomes, e.g., Rab5, Rab4, Rab11, Rab7, Rab17, Rab18, Rab22, as a whole in order to establish various (GTPase) effector/regulator proteins that are suitable as targets for pharmaceutical drugs. In this approach, the GTPase itself will not serve as a target molecule because its activity is so crucial for cell homeostasis that it would likely invariably result in toxic effects. Rather, the effector/regulator molecules specifically functioning in protein and lipid sorting, endosome motility and endosome-cytoskeleton interactions will be the target. For example, compounds that affect cell motility could be screened more specifically without affecting other important cellular functions (i.e. endocytosis). In the alternative, and according to a preferred embodiment of the present invention, the target may well be one of the effectors/regulators as defined above, but additionally a GTPase such as Rab5, Rab4, Rab7, Rab11, or Rab17, will be present in the assay system.

The present inventors have functionally characterised several Rab GTPases (e.g., Rab5, Rab4, Rab11, Rab 7, Rab17, Rab18, Rab20) and effectors/regulators of these GTPases, localised to the early endosomes and the recycling endosomes, which GTPases and effectors/regulators, respectively, are thus suitable for the assay according to the invention. These Rab proteins sequentially control transport in the endocytic and recycling pathway. Rab5 controls transport from the plasma membrane to early endosomes whereas Rab4 and Rab11 function in protein sorting and recycling from early and recycling endosomes back to the plasma membrane. The expression of Rab17 is restricted to polarised cells such as epithelial cells and neurons. This latter GTPase regulates trafficking through the apical recycling endosome, thus contributing to the generation and maintenance of cell polarity. Thus, effectors/regulators of all these GTPases and the respective GTPases (in combination with their effectors/regulators) may be employed in accordance with the present invention in an *in vitro* or *in vivo* assay system as a target in order to screen for substances that are useful as a pharmaceutical agent for the prophylaxis and treatment of the above mentioned diseases, i.e., cancer (benign tumours, malignant tumours, carcinomas, sarcomas, melanomas, leukemias, gliomas, or neuroblastomas), diabetes, Alzheimer's disease, endometriosis, atherosclerosis, allergic, inflammatory, and infectious diseases such as AIDS, tuberculosis, cholera, malaria, pseudotuberculosis, gastroenteritis, enteric fever, typhus, and any other infectious diseases caused by an infectious agent that infects cells by the endocytic route and resides intracellularly in phagosomes escaping the cellular killing mechanisms (in this regard, it should be recalled that the GTPases per se and alone can not serve as a target for the drugs to be detected; rather, the target for the drug is an effector/regulator molecule). Other examples of infectious diseases are those diseases caused by the following pathogens (bacteria or organisms): Mycobacterium, Staphylococcus, Toxoplasma, Trypanosoma, Listeria, Salmonella, Legionella, Leishmania, Coxiella, Shigella, Yersinia, Neisseria, Vibrio, Bartonella, and any other intracellular pathogen that resides in intracellular phagosomes and escapes cellular killing mechanisms.

As the proteins described herein, i.e., the Rab proteins and their effectors/regulators, are, unless exceptions, ubiquitously expressed, pharmaceutical agents for the prophylaxis and treatment of tumours of any cellular origin can be screened for and detected according to the present invention. Specifically, the following tumours may be mentioned: lung carcinoma, osteosarcoma, lymphoma, leukemia, soft tissue sarcoma, breast carcinoma, bile cancer, cervix carcinoma, cancer of the (small) intestine, of the kidneys, of the cavity of the mouth, penis carcinoma, ovary cancer, stomach cancer, cancer of the tongue, brain cancer, bladder carcinoma, prostate

carcinoma, liver carcinoma, and carcinoma of the pancreas and every tumor that invades other tissues and organs.

Drugs with anti-cancer and/or an activity against any of the other above mentioned diseases include those molecules (including peptides) mimicking the peptide structure of the respective target molecule. Typical representatives of the above classes are molecules/substances that carry one or more of the following functional groups: a halide atom bound to an alkyl, alkenyl, alkynyl or aryl residue, an alcohol group (primary, secondary, tertiary), an ether group, a carbonyl function (aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group including a lactone group, a benzyl, phenyl, tolyl, tosyl, sulfonyl group, an amino group (primary, secondary, tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group, and a quinone group. Other representatives of suitable drugs are organometallic compounds, sterol moiety(ies)-containing molecules,  $\beta$ -hydroxy carboxylic acids, inorganic acids, and complexes such as metallocenes; nucleic acids, cytokines, hormones, antibodies, or oligopeptides comprising up to 20, preferably 8, 10, or 12, amino acid residues. Effectors transmit the function of the GTPase, Rab is upstream and recruits the effectors which do the work. Regulators instead switch the Rab on and off. Rab-on (=GTP) binds the effectors and is functional.

To elucidate the molecular mechanism whereby these GTPases exert their function in membrane trafficking and cytoskeleton organisation, the present inventors have developed a method to biochemically identify Rab5 protein effector molecules (Chistoforidis et al., 1999a). This method is based on previously described affinity chromatography (Amano et al., 1996) but has been optimised to result in the specific elution and large-scale purification of Rab effectors and GTPase interacting proteins in general, in amounts sufficient for both their identification by microsequencing techniques and their functional characterisation (Chistoforidis et al., 1999a).

In addition to that method, the present inventors have developed a novel method. This method is described in Example 3 below. With this method, the inventors have re-purified several of the proteins that bind Rab5. Using this modified affinity chromatography procedure, more than 20 effector and/or regulator proteins interacting directly or indirectly with Rab5 (Figure 1a in (Chistoforidis et al., 1999a)) have been identified and demonstrated to exhibit functional activity as shown by their ability to substitute cytosol in an *in vitro* endosome fusion reaction (Chistoforidis et al., 1999a). Some of these molecules such as Rabex-5 regulate the activity of

Rab5 (i.e., its nucleotide state) (Horiuchi et al., 1997). Others such as Rabaptin-5 and EEA1 participate in the endosome docking and fusion process (Stenmark et al., 1995). Still others regulate the ability of endosomes to move along microtubules, and all of these proteins are suitable candidates as a target for the detection of chemical drugs (compounds) that may turn out to be highly efficient agents to combat the above mentioned diseases.

In addition, expression of an activated mutant of Rab5 (Rab5Q79L) induces an increased recruitment of actin on the expanded endosomes suggesting that Rab5 either directly or indirectly regulates the attachment/nucleation of actin filaments with the endosome membrane. Based on the above data, in particular in view of this large number of Rab5 interacting molecules (more than 20), the present inventors concluded that the activity of Rab5 is not restricted to endocytic membrane docking and fusion. Most importantly, they also concluded that modulating the activity of Rab5, and therefore of its effectors/regulators, would result in changes in organelle motility *in vivo* and in cell motility. This is perfectly consistent with the effect observed by the present inventors that expression of activated Rab5 leads to an increase in motility of fibroblasts and endothelial cells, implicating this GTPase in the regulation of cell migration. The effector/regulator protein(s) responsible for this activity (to regulate membrane trafficking and membrane-cytoskeleton interactions including the ability of endosomes to move along microtubules) are therefore, in accordance with the present invention, specific targets for drugs aimed at inhibiting the ability of the Rab5 machinery to sustain cell migration.

Similar experiments have been and are being conducted on two other GTPases functioning in endosome trafficking, Rab4 and Rab11. Several candidate proteins for Rab4 effectors/regulators have been purified and one of these proteins has been sequenced and identified as the human homologue of the yeast Adenyl Cyclase-associated protein CAP (Field et al., 1990). This protein is a component of the Ras-activated cyclase complex in yeast and therefore is required for the proliferative signal in yeast. In addition, this protein has been implicated in the interaction with the actin cytoskeleton, since the carboxy terminus of CAP binds actin. Another protein which has been identified is Rabenosyn-5, a Rab5 effector. The realisation that this molecule binds both Rab5 and Rab4 but does not interact with Rab11, suggests that it may control trafficking between the Rab5 and Rab4 sub-compartments within early endosomes, and therefore regulate the sorting of proteins trafficking through this organelle. This hypothesis is consistent with the new experimental data recently obtained (see below).

Given the role of Rab proteins in the regulation of membrane transport and membrane-cytoskeleton interactions, the effectors/regulators for these small GTPases are targets for pharmaceutical drugs aimed at (1) inhibiting cell invasiveness and (2) enhancing cell-defence mechanisms against intracellular pathogens, such drugs therefore being valuable weapons in the battle against tumor (and cancer) and all other of the above mentioned diseases. For example, it may be possible to inhibit the stimulatory activity of Rab5 to reduce cell motility and consequently to reduce metastasis and the progression of cancer.

A strategy similar to that established and described above for Rab5 has been applied for other Rab proteins regulating endocytic trafficking. For example, the small GTPases Rab4, Rab11, and Rab17 reside onto and regulate trafficking through the early and recycling endosomes. Given that in motile cells recycling endosomes preferentially deliver membranes to the leading edge of the cell and given that the deposition of adhesion molecules to the leading edge of motile cells depends on the endocytic cycle between endosomes and the plasma membrane, the established functional role of Rab proteins functioning on endosomes, including Rab4 and Rab11 characterised by the present inventors, implies that they may participate in the regulation of cell motility. Therefore, the effectors/regulators of these and other endosomal GTPases will also represent novel potential targets according to the instant invention.

On the other hand, further Rab effectors/regulators to be identified and characterised in the future will also act in various aspects of endosome function, including endosome and cell motility, and endosome-phagosome interactions. An approach to identify further effectors/regulators has been described (Chistoforidis et al., 1999a), and the procedure described by the authors is similarly suitable to detect, identify, and characterise further, as yet unknown, effector/regulator molecules (proteins) of small GTPases of the Rab family.

The multiplicity of Rab5 (or any other Rab GTPases) effector/regulator molecules serves to execute distinct functions in endosome dynamics and cell dynamics all coordinated by the GTPase switch of Rab5 (or any other of the GTPases). It is therefore a “package” also referred above as a network of molecules that is necessary to coordinate different functions. These molecules can operationally be divided into the following classes on the basis of either their established identity and/or function and on the basis of their possible function for which different claims in drug discovery can be proposed. The effectors/regulators of all classes may be used for



the assay as defined herein, i.e., for the detection of suitable drugs for the treatment of any of the above mentioned diseases.

Class 1: Representative effectors/regulators: Rabaptin-5, Rabaptin-5b, Rabex-5, RN Tre, EEA1, PI 3-K, Inositol Polyphosphate 4-phosphatase and Inositol Polyphosphate 5-phosphatase.

Function: endocytic trafficking

Assay: endosome fusion, endosome motility

This class of Rab5 effectors/regulators includes molecules that 1) have either been already described as functionally linked with Rab5 (Rabaptin-5, Rabaptin-5b, Rabex-5, EEA1, PI 3-K), or 2) have previously been identified and whose DNA and deduced protein sequence have been disclosed (RN Tre, Inositol Polyphosphate 4-phosphatase and Inositol Polyphosphate 5-phosphatase) but that have been now functionally linked to Rab5 by the present inventors. Rab5 effectors/regulators of this class 1 belong to the “package” also referred above as a network of molecules that is necessary to coordinate different functions of Rab5.

Since Rab5 regulates endocytosis, effectors/regulators of this molecule also control endocytosis as well as all functions controlled by Rab5; i.e., endocytic trafficking, intracellular distribution of endosomes, motility of endosomes along microtubules, and cellular motility. Consequently, class 1 Rab5 effectors should be considered potential targets for the therapeutic strategies above described in conjunction with the other classes of Rab5 effectors/regulators.

Class 1 includes

1) Regulators that play a role in the nucleotide cycle of Rab5. Rabex-5 has been identified as a nucleotide exchange factor for Rab5 (Horiuchi et al., 1997), and therefore converts the GTPase from the inactive, GDP-bound, to the active, GTP-bound form. Consequent to the activity of Rabex-5 is the interaction between Rab5 and the Rab5 effectors that transmit Rab5 function. The present inventors have discovered that RN Tre (Matoskova et al., 1996), a protein related to the *Tre* oncogene, is a Rab5 GAP, which discovery provides an interesting link between the signal transduction and membrane trafficking machinery. Most importantly, like Rabex-5 that regulates activation of Rab5, RN Tre regulates its inactivation and therefore represents an ideal target for drugs aimed at interfering (positively and negatively) with Rab5 function.

2) Effectors that play a role in endosome docking and fusion: Rabaptin-5 (Stenmark et al., 1995), Rabaptin-5b (Gournier et al., 1998), and EEA1 (Christoforidis et al., 1999a; Simonsen et al., 1998), or in the regulation of this process via the generation of phosphoinositide-3-phosphate (PI 3-K) which is necessary for the membrane recruitment of EEA1 (Christoforidis et al., 1999b).

5 Two distinct phosphatidyl-inositol 3-kinases (PI 3-K), hVPS34 and p85a/p110b, have been identified among the Rab5 effectors. The two kinases are differentially distributed along the endocytic pathway, and the activity of hVPS34 is specifically required for EEA1 membrane recruitment and endosome fusion. It has also been demonstrated that VPS34 regulates the motility of endosomes along microtubules, suggesting that PI 3-K activity is necessary to  
10 coordinate the function of various Rab5 effectors/regulators, including those controlling membrane-cytoskeleton interactions (Nielsen et al., 1999). PI 3-K are therefore considered to represent a particularly suitable target of drugs that block the motility activity. Similarly, targets other than PI 3-K may be identified among the Rab5, Rab4, and Rab11 effectors/regulators that couple the activity in membrane docking and fusion with the organelle dynamics. One such  
15 target coupling the activity in membrane docking and fusion with the organelle dynamics is Rabaptin-5 due to its capacity to couple Rab4 to Rab5 function.

The present inventors have determined that the list of Rab5 effectors includes two more proteins that play a role in phosphoinositide metabolism: Inositol Polyphosphate 4-phosphatase and  
20 Inositol Polyphosphate 5-phosphatase. First tests conducted by the present inventors have proven that both these phosphatases bind to a Rab5 affinity column and directly interact with Rab5 in a yeast two-hybrid system assay. The function of Inositol Polyphosphate 4-phosphatase and Inositol Polyphosphate 5-phosphatase is to catalyse the removal of the phosphate from the 4- and 5-position of the inositol ring, respectively. In the context of the Rab5 network of  
25 effectors/regulators, these proteins are functionally linked to the PI3-Ks, especially p85a/p110b, since Inositol Polyphosphate 4-phosphatase and Inositol Polyphosphate 5-phosphatase would use as substrate the product of this kinase. The discovery that 1) Rab5 regulates a sophisticated cycle of phosphorylation/dephosphorylation of phosphoinositides and 2) that phosphoinositides regulate the recruitment of factors playing a role in the endosome transport machinery as well as  
30 signaling molecules, implies that Rab5 and its effectors/regulators can be used as drug targets for diseases where alterations in the function of endosomes can result in therapeutic intervention.

The class 1 effector/regulator molecules may be used according to the present invention as drug targets in cancer on the basis of regulating cell motility and invasiveness. The inventors of the  
35 present invention have found out that Rab5 regulates the motility of endothelial cells in vitro (this result was the outcome of experiments described in the Examples below). Since the

Rabaptin-5/Rabex-5 complexes regulate the nucleotide cycle of Rab5, and since the kinases PI 3-K regulate the recruitment of effectors/regulators, these molecules have been identified as targets for drugs aimed at inhibiting the activity of Rab5 in cell motility. The class 1 effector/regulator molecules may also be used as drug targets in cancer on the basis of the signalling molecules regulating cell growth. Growth factors and growth factor receptors are transported through the endosomes before being degraded in the late endosomes and lysosomes. Molecules that regulate the trafficking of receptors along the endocytic pathway can be used as targets for drugs aimed at interfering with transport (i.e., clearance from the plasma membrane, inhibition of recycling, transport to endosomes where signalling molecules are located, increase transport to degradative compartments where receptor-ligand complexes are degraded) and, consequently, inhibiting mitogenic signalling. Finally, the class 1 effector/regulator molecules may also be used as drug targets in infectious diseases (see the above list exemplifying infectious diseases particularly suitable according to the present invention). The small GTPase Rab5 has been observed to accumulate on *Mycobacterium tuberculosis* phagosomes. Interestingly, the Rab5 effector Rabaptin-5 is not present on the phagosome membrane. Thus, it must be concluded that there are differences between endosomes and phagosomes in the utilisation of the Rab5 effectors/regulators. In the case of *Listeria monocytogenes* phagosomes, it has been observed that an upregulation in expression of the small GTPase Rab5 occurs in the course of infection of macrophages by a *Listeria monocytogenes* non-lytic mutant. This increased Rab5 recruitment results in an increased fusogenicity of the phagosomes with endosomes leading to intracellular killing of the bacterium. Accordingly, in order to detect and identify substances suitable for the therapeutic treatment of pathogen infections, the Rab5 effector/regulator responsible for this effect may be used as a target in the assay described in detail further below. In this case, the upregulation of the host cell defence mechanism could be accomplished by a suitable drug. The activator of Rab5, Rabex-5, that functions as a nucleotide exchange factor and converts the protein into the GTP-bound active form (Horiuchi et al., 1997) is a particularly suitable drug target in this regard. By enhancing its activity (by means of a drug activating Rabex-5), the inventors have been able to up-regulate the Rab5 machinery, stimulate endosome fusion and facilitate phagosome-endosome interactions. Alternatively, other Rab5 effectors/regulators (still to be identified) playing a role in endosome trafficking could serve as targets.

Class 1 as well as the other classes of Rab5 effectors described below may include molecules playing a role in endosome motility. The present inventors have found that Rab5 regulates the motility of early endosomes *in vivo* and both the attachment of early endosomes to microtubules

and the motility of early endosomes along microtubules *in vitro* (Nielsen et al., 1999). New data is that a Rab5 eluate is sufficient to reconstitute motility in an endosome-microtubule *in vitro* motility assay and in the absence of cytosol. Therefore a Rab5 effector(s) is/are sufficient to regulate or switch on a microtubule motor, or a Rab5 effector(s) is/are a microtubule motor.

5 For this function we make three statements to support a claim:

1. Rab5 is required to bind certain or possibly all early endosomes to microtubules.
2. Rab5 effectors and possibly their binding partners (i.e. a Rab eluate) are required for endosome motility on microtubules.
3. RabGDI blocks all endosome motility *in vivo* (microinjection of RabGDI).

10 These data demonstrate an essential role for Rab5 effectors in binding endosomes to microtubules and their subsequent motility along microtubules. Rab5 effectors can therefore be used as target molecules for drugs aiming to reduce endosome motility to block endosome cycling and, consequently, recycling of surface molecules necessary for cell motility.

## 15 Class 2

Representative effectors/regulators: Rabankyrin-5 and Rabenosyn-5 (p110 FYVE)

Function: endocytic trafficking, possible interaction with the actin cytoskeleton .

Assay: endosome fusion, endosome motility

20 This class includes at least two proteins which share the same PI(3)P-binding domain, the FYVE-finger. One protein is the human homologue of mouse Ankhzn (Ito et al., 1999), to be renamed Rabankyrin-5, given its ability to bind Rab5. The present inventors also found a Rabankyrin-5 splice variant (SV). Rabankyrin-5 contains ankyrin repeats. It is localised to early endosomes, binds Rab5, plays a role in endosome fusion. and is required for early endosome  
25 fusion. As regards the binding to Rab-5, it is worth noting that the present inventors determined the Rab5 binding region to reside in the ankyrin repeats in the C-terminus of the protein (between residues 542 and 1075).

The sequence of the gene coding for human Rabankyrin-5 is depicted in the sequence listing  
30 attached to this application (SEQ ID NO: 1) whereas the sequence of the protein itself is given in the sequence listing as SEQ ID NO: 2. The nucleotide and amino acid sequences of the SV gene and protein are indicated in the sequence listing as SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

To the best knowledge of the present inventors, there is no prior art that would suggest a role for the gene/gene product of ankhzn - or of its human analogues - as a Rab5 effector with a role in endosome fusion and membrane trafficking. The second molecule is Rabenosyn-5, previously named p110FYVE. It is localised to early endosomes as well, binds Rab5 as well, and is also known to be complexed with and bind to the mammalian homologue of yeast Vps45p, a protein involved in transport to the vacuole. This binding demonstrates that Rabenosyn-5 has a role in SNARE-mediated membrane fusion. Rabenosyn-5 (p110FYVE) probably also plays a role in the regulation of transport and participates in the movement of endosomes along the actin and microtubule cytoskeleton.

The nucleotide and amino acid sequences of p110FYVE are given in SEQ ID NO: 5 and SEQ ID NO: 6, respectively. Further properties of the effector Rabenosyn-5 (p110 FYVE) are that it is essential in endosome fusion, fusion of clathrin coated vesicles derived from the plasma membrane with early endosomes. Over-expression of Rabenosyn-5 inhibits the processing of lysosomal enzymes.

The present inventors have found that Rabenosyn-5 is a Rab4 effector. It has been known that three small GTPases, i.e., Rab5, Rab4, and Rab11, exist in separate domains on the same endosome. The present inventors have been able to demonstrate that over-expression of Rabenosyn-5 brings the Rab5 and Rab4 domains together, without changing the distribution of Rab11, and changes the sorting properties of the endosome with respect to the cycling of certain receptors (e.g. transferrin receptor). This proves that Rabenosyn-5 has a function in receptor sorting and provides validation that manipulating Rabenosyn-5 has the potential to control receptor amount, distribution, and the kinetics of receptor cycling.

### Class 3

Representative effectors/regulators: multi-protein complex including p100, p95, p60, p45, p25

Function: endocytic trafficking, possibly in polarised cells such as epithelial cells and neurons

Assay: endosome fusion, endosome motility

This complex consisting of the above 5 proteins (p100, p95, p60, p45, p25) and exhibiting the molecular weights of 100 kDa, 95 kDa, 60 kDa, 45 kDa, and 25 kDa, respectively, has been isolated by the present inventors. At least one of these molecules, or the entire complex, interacts either directly with Rab5 or indirectly through another Rab5-binding protein. The present

inventors have also determined that the complex is detected in a clathrin coated vesicles-enriched fraction. This subcellular fraction includes the vesicles that transport protein receptors from the plasma membrane to the early endosomes.

- 5 The structure (sequence) and function of the 5 protein components have been investigated to some extent and are more or less known now and form part of the present invention.

In detail, the C-terminal half of the 100 kDa component shares sequence homology (52%) with the conserved catalytic domain of GTPase activating proteins and spindle assembly checkpoint  
10 proteins from yeast to mammals, while the N-terminal half shares homology (57%) with the conserved domain of serine/threonine protein kinases. Fusion between early endosomes is strongly inhibited when cytosol is immuno-depleted using anti-45 kDa component antibodies.

Immunofluorescence data show that the 45 kDa component is localised in intracellular structures  
15 which are not EEA1 positive. Upon over-expression of Rab5Q79L though, there is partial co-localisation of these proteins on early endosomes.

The nucleotide and amino acid sequences of these proteins have been determined and are depicted in the attached sequence listing (p60: SEQ ID NO: 7 and SEQ ID NO: 8; p45: SEQ ID  
20 NO: 9 and SEQ ID NO: 10; p25: SEQ ID NO: 11 and SEQ ID NO: 12, p100: SEQ ID NO: 13 and SEQ ID NO: 14; p95: SEQ ID NO: 15 and SEQ ID NO: 16, respectively). The p95 sequences are only partial sequences, however.

## Examples

### 25 Example 1:

The following example describes the materials and methods used to measure the effect of a GTPase or mutant GTPase on the regulation of cell motility.

### *Expression constructs*

30 Myc-rhoDV26G (Murphy et al., 1996) was cloned into the HindIII-XbaI sites of expression vector pHSVPUC (Geller et al., 1993) to generate construct: pHSV-myc-rhoDV26G. Myc-rhoDV26G cDNA was cloned in frame into the EcoRI-BamHI sites of expression vector pEGFP-C2 (Clontech) to generate a green fluorescent protein (GFP) fusion: GFP-myc-RhodV26G.

*Transient transfection of rhoDV26G and human transferrin receptor*

BHK-21 cells were trypsinized 24h prior to transfection and were seeded onto 11mm glass coverslips. Cells were infected with T7 RNA polymerase recombinant vaccinia virus (Bucci et al., 1992) and transfected with either T7-myc-rhoDV26G and T7-human transferrin receptor (T7-hTR) or T7-hTR alone (Murphy et al., 1996) using DOTAP (Boehringer Mannheim, Mannheim, Germany). The cell number and also the amount of DNA and lipid were constant. The final concentration of DNA per coverslip was 1µg.

Following transfection, the cells were incubated at 37°C, 5% CO<sub>2</sub>, for 2h, cyclohexamide was then added for 1h. Cells were washed 8 times for 1min in preheated medium and rhodamine transferrin (50 µg/ml) was uptaken in a preheated humidified chamber at 37°C, 5% CO<sub>2</sub>, for 20min. Coverslips were washed in preheated medium and then mounted into chambers for video microscopy (Bradke and Dotti, 1997; Bradke and Dotti, 1996) as described below. TLVM (time lapse video microscopy) was carried out immediately. Hydroxyurea was present at all times to prevent late viral gene expression.

*Transient transfection of T7-rhoDV26G and T7-gfp*

To monitor lysosomal motility in the presence and absence of rhoDV26G we made use of a green fluorescent protein-expressing vector cloned into pGEM1 harbouring the T7 promoter. Co-transfection of this plasmid with T7-myc-rhoDV26G allowed us to identify the transfected cells for video microscopy. Handling of the cells was as outlined above with the exception that they were incubated in the presence of 50 nM LysoTracker (Molecular Probes) for 15min at 37°C, 5% CO<sub>2</sub> to label the lysosomes, and TLVM was carried out as outlined below.

*BBCE cell motility*

To address the effect of rhoDV26G on cell motility, BBCE cells were trypsinized and plated at a density of 1,500 cells per cm<sup>2</sup>, 24h later nuclear microinjection was carried out with a RhoDV26G expression construct at 50µg/ml concentration, FITC dextran was coinjected to identify the injected cells. Approximately, 12h following injection the cells were stimulated with bFGF and subjected to TLVM.

*Time-Lapse Video Microscopy*

The cells for TLVM were grown on coverslips and mounted into aluminum chambers as described in (Bradke and Dotti, 1997). Briefly, aluminum was cut into 7.5cm x 2.5cm x 2mm rectangles, the dimensions are similar to a standard glass slide and fit onto the microscope stage. In the centre a circular hole was cut of 8mm diameter, and around it 4mm area was milled from both sides. A glass coverslip was inserted onto one side of the chamber, sealed with a lubricant, medium was then added to fill the chamber, cells grown on coverslips were then inverted onto the other side of the metal slide and sealed with lubricant.

#### *Image and statistical analysis of the data*

A dedicated automatic program was developed to detect and track endosomes/lysosomes as they move. It runs on a SPARC station Ultra1 (SUN, Mountain View, CA) to which a Series 151/40 digital image processor (Imaging Technology, Bedford, MA) is connected. The detection of fluorescent spots corresponding to endosomes/lysosomes is performed automatically by a multiresolution algorithm based upon selectively filtering an undecimated wavelet decomposition of the image through the use of wavelet coefficient thresholding and correlation. At the end of this step, all endosomes/lysosomes in the sequence are characterised and their coordinates are determined and stored. A tracking algorithm is then used to establish valid trajectories. The algorithm uses a first-order Kalman filtering approach whereby at each frame and on the basis of the previous ones, predictions of the endosome/lysosome positions are established and compared with the computed ones. The best matches are selected as trajectory points and tracks are finally analysed to compute the data that was used for generating values in Tables 1 and 2. For each data set, a sequence of 30 images was analysed. Speed values reported here are mean values of the speed of several endosomes/lysosomes and errors are standard deviations of these group estimated.

#### Example 2:

Drugs that have been found by employing the assay of the present invention and that enhance or inhibit Rab5 effector/regulator activity can be distinguished and classified on the basis of their mode of action (activities 1 to 5, see below). Accordingly, 5 classes of drugs will be introduced (and searched for) that exhibit a specific mode of action on the target molecules, i.e., on the effectors or regulators:

1. Drugs that inhibit Rab5 activation. Such drugs would in principle act on the whole Rab5 network and are therefore less specific than drugs of classes 3 and 4 because they act on a



regulator (protein or molecule) that affects the nucleotide state of Rab5 and, consequently, the ability of Rab5 to signal to the totality of Rab5 effectors rather than on a specific effector (protein or molecule).

2. Drugs that enhance Rab5 inactivation. Such drugs would in principle act on the whole Rab5 network and are less specific than drugs of classes 3 and 4 for the reason given for class 1 above.

3. Drugs that disrupt specific Rab5-effector interactions, e.g., between Rab5 and p110 FYVE.

4. Drugs that inhibit a specific Rab5 effector activity, e.g., endosome fusion, endosome-microtubule interaction, endosome motility along microtubules.

5. Drugs that inhibit cell motility.

Class 3 and 4 drugs are the most specific and are therefore preferred according to the present invention. However, drugs of the other classes, in particular of classes 1 and 2, can nevertheless be considered as similarly suitable agents to combat at least one of the above mentioned diseases.

#### Assay for Activity 1

The assay measures Rabex-5 dependent nucleotide exchange of Rab5. The drug could perturb Rab5-Rabex-5 interaction or inhibit the catalytic activity of Rabex-5. The result expected is an inhibition of GDP/GTP exchange of Rab5. This assay is based on the assay published in (Horiuchi et al., 1997) and may well be adequately adapted for high throughput screening. It will be readily feasible, therefore, to screen thousands of compounds for their potential anti-cancer or anti-infectious disease activity/potential.

GDP/GTP exchange activity. Unless otherwise specified, the standard [<sup>35</sup>S]GTPγS-binding assay was performed by the filter method (Sasaki et al., 1990) by incubating 200nM of recombinant Rab5 with 1μM of [<sup>35</sup>S]GTPγS (20,000cpm/pmol) at 37°C for 10min with 5mM of MgCl<sub>2</sub> in the presence of 20nM recombinant Rabex-5.

Substances that are able to enhance or inhibit nucleotide exchange above or below the standard activity measured in the presence of Rabex-5, respectively, may be searched for.

#### Assay for Activity 2

The assay measures the GTPase Activating Protein (GAP) RN-Tre-dependent stimulation of GTP hydrolysis by Rab5. The drug could stabilise the Rab5-GAP interaction or stimulate the

catalytic activity of GAP. The result expected is an increase in the inactive-form of Rab5, i.e., an increased amount of GDP-bound Rab5. This assay is based on the following assay:

GAP assays with purified recombinant RN-Tre fusion proteins were performed by the filter method (Sasaki et al., 1990) using 2 $\mu$ M of substrate [ $\gamma$ -<sup>32</sup>P]-GTP-loaded Rab5 in the presence of recombinant RN Tre GAP domain (100nM), at 30°C for 2min. GAP activity was expressed as the percentage of non-hydrolysed [ $\gamma$ -<sup>32</sup>P]-GTP which remained bound to the filters, relative to the radioactivity at time 0. Substances that are able to increase the GTP hydrolysis activity compared with the standard activity measured in the presence of RN Tre should be searched for.

The assay may adequately be adapted for high throughput screening.

### Assay for Activity 3

Drugs may be screened for their ability to inhibit specific Rab5-Rab5 effector interactions. The assay consists in the binding of an effector molecule (either *in vitro* translated or as purified recombinant protein; native or modified, e.g., by biotinylation) to GST-Rab5:GTP $\gamma$ S (GTPgammaS) immobilised on a matrix. This assay is based on the procedure developed by Christoforidis et al. (1999a and b), but may be readily adapted for high throughput screening.

GST-Rab5 affinity chromatography is performed as described previously (Christoforidis et al., 1999a). Beads with GST-Rab5 were incubated with the nucleotide exchange buffer (NE buffer) containing 20mM Hepes, 100mM NaCl, 10mM EDTA, 5mM MgCl<sub>2</sub>, 1mM DTT, 1mM GTP $\gamma$ S, pH7.5, for 90min at room temperature under rotation. Afterwards, NE buffer was removed and the nucleotide was stabilised with NS buffer containing 20mM Hepes, 100mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT, 1mM GTP $\gamma$ S, pH7.5, for 20min at room temperature under rotation. *In vitro* transcription-translation of <sup>35</sup>S-methionine labeled proteins is performed according to Manufacturer's instructions (Promega). Recombinant proteins or transcribed-translated proteins (50 $\mu$ l of standard reactions) are incubated for 2h at 4°C with 20 $\mu$ l glutathione sepharose beads coupled with GST-Rab5:GTP $\gamma$ S, and 150 $\mu$ l buffer containing 20mM Hepes, 100mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT, and 1mM GTP $\gamma$ S, respectively, such that the final volume is 220 $\mu$ l. The incubation is performed both in the presence and in the absence of the candidate drug. When the drug is dissolved in an organic solvent the buffer in the control reaction will be supplemented with the solvent in the same concentration and amount added in the reaction in the presence of the drug. Usually, however, the buffer/solvent dissolving the drug is the same buffer used for the

assay, i.e., the buffer containing 20mM Hepes, 100mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT, and 1mM GTPγS. Beads are subsequently washed 4x with buffer containing 20mM Hepes, 100mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT, 10μM GTPγS, 1x with the same buffer but now containing 250mM NaCl, and 1x with 20mM Hepes, 250mM NaCl, 1mM DTT. Elution of bound proteins is performed as described before (Christoforidis et al., 1999a; Christoforidis and Zerial, 1999c) and eluted proteins are loaded on SDS-PAGE gel followed by immunoblotting for recombinant proteins or autoradiography for *in vitro* translated proteins.

The assay measures the activity of drugs that are capable of disrupting the association of a specific or any Rab5 effector with Rab5 in a concentration-dependent manner under the same experimental conditions that allow the interaction. For example, a drug x will disrupt the interaction of effector X with Rab5:GTPγS in a concentration-dependent manner. This drug will then be used as inhibitor of effector X in the biological process X.

A variation of the assay is to use magnetic beads in an assay of the ELISA-type (similar to the *in vitro* endosome fusion assay described below). GST-Rab5 was biotinylated using chemical crosslinking (Pierce) and nucleotide exchange was performed. Beads with GST-Rab5 were incubated with NE buffer containing 20mM Hepes, 100mM NaCl, 10mM EDTA, 5mM MgCl<sub>2</sub>, 1mM DTT, 1mM GTPγS, pH7.5, for 90min at room temperature under rotation. Afterwards, the NE buffer was removed and the nucleotide was stabilised with NS buffer containing 20mM Hepes, 100mM NaCl, 5mM MgCl<sub>2</sub>, 1mM DTT, 1mM GTPγS, pH7.5, for 20min at room temperature under rotation. Beads (1μl) were subsequently incubated with 1μg recombinant purified Rab5 effector coupled to a ruthenium trisbipyridine chelate (IGEN) in 20μl NS buffer in the presence or absence of chemical drug candidates. Beads were then washed three times with 500μl NS buffer and binding of the effector to Rab5 is measured with an Origen Analyzer (IGEN) according to the manufacturer procedure. The background signal (binding to Rab5:GDP, i.e., without nucleotide exchange) is deducted. Drugs that affect the interaction between Rab5 and the Rab5 effector decrease the signal in comparison with the signal obtained when the reaction is performed in the absence of the drug.

The assay measures the activity of drugs that are capable of disrupting the association of a specific or any Rab5 effector with Rab5 in a concentration-dependent manner under the same experimental conditions that allow the interaction. For example, a drug x will disrupt the

interaction of effector X with Rab5:GTPgS in a concentration-dependent manner. This drug will then be used as inhibitor of effector X in the biological process X.

#### Assays for Activity 4

- 5 The present inventors have developed three main assays to measure Rab5 and Rab5 effector activity.

##### (i) *In vitro* fusion assay (Horiuchi et al., 1997)

- 10 This assay is intended to screen for drugs that inhibit endocytosis, clathrin coated vesicle (CCV) fusion with early endosomes, early endosome homotypic fusion and endosome trafficking in tumor cells or enhance endocytic membrane fusion in bacterial parasites infected cells.

- Two distinct enriched populations of early endosomes labeled with either biotinylated transferrin or a sheep  $\alpha$ -human transferrin antibody are prepared from sHeLa (suspension HeLa) cells by sucrose sedimentation (Gorvel et al., 1991). The basal fusion reaction consists of the two enriched endosome populations incubated for 45minutes at 37°C in the presence of 3mg/ml of HeLa cytosol, unlabelled transferrin and an ATP regeneration system (17.3mM creatine phosphate, 87 $\mu$ g/ml creatine kinase, and 2.2mM ATP) and in the presence or absence of the candidate drug. The fusion is quantified by incubation of the fusion mix with wash buffer (50mM Tris pH7.5, 100mM NaCl, 1g/l BSA and 2% (w/v) Triton X-100) and streptavidin-coated magnetic beads (Dynal). After two washes in wash buffer the samples are incubated with a rabbit  $\alpha$ -sheep secondary antibody coupled to a ruthenium trisbipyridine chelate (IGEN) and measured with an Origen Analyzer (IGEN). The background signal (5-10% of the fusion signal) is deducted and the data expressed as the percentage of the basal fusion reaction. The CCV-early endosome fusion assay is performed in the same way with biotinylated transferrin-labeled CCV and  $\alpha$ -transferrin antibody-labeled early endosomes.

- Substances that are able to inhibit CCV-endosome or early endosome fusion below the standard fusion activity in the presence of cytosol and an ATP-regenerating system may be searched for.
- 30 To control that the inhibition is targeted to a Rab5 effector (by inhibiting the Rab5-Rab5 effector interaction or the Rab5 effector activity), the inhibitory effect of the drug should be rescued by increasing concentrations of that particular Rab5 effector. For this a titration curve of the drug in the presence of increasing concentrations of the Rab5 effector will be necessary.

The following two assays are intended to screen for drugs that inhibit endosome movement and cell motility in tumor cells or enhance endocytic membrane fusion in infected cells. The assays score the overall activity, but as soon as the effector molecules specifically involved in these processes are identified, it will provide the additional possibility of using Assay for activity 3.

(ii) Microtubule spin-down assay

75 to 100µg purified early endosomes are incubated at room temperature for 20min with 100µg HeLa cytosol protein in a reaction brought to a final volume of 50µl by addition of BRB-80 (80mM K-Pipes, 1mM MgCl<sub>2</sub>, 1mM EGTA, pH6.8). 10µl of taxol-stabilized microtubules (100µg tubulin equivalent) are added, and this mix is incubated for 10min at room temperature, in the presence or absence of the candidate drug, and then overlaid on 600µl of a 35% (w/v) sucrose cushion. After sedimentation at 100,000 x g for 20min at 22°C in a TLA 100.4 rotor, the upper layer was removed, the cushion was washed with 100µl BRB-80 and then also removed. The remaining pellet was resuspended in 30µl 2x SDS-PAGE buffer plus 30µl dH<sub>2</sub>O, and analysed by SDS-PAGE and immunoblotting.

Substances that are able to inhibit the association of early endosomes with microtubules below the standard fusion activity in the presence of cytosol and an ATP-regenerating system may be searched for. To control that the inhibition is targeted to a Rab5 effector (by inhibiting the Rab5-Rab5 effector interaction or the Rab5 effector activity), the inhibitory effect of the drug may be rescued by increasing concentrations of that particular Rab5 effector. For this a titration curve of the drug in the presence of increasing concentrations of the Rab5 effector is necessary.

(iii) *In vitro* endosome motility assay

Early endosomes and cytosol are isolated as previously described (Horiuchi et al., 1997), except that HeLa spinner culture cells were allowed to internalise rhodamine-labeled transferrin for 10min at 37°C in order to label early endosomes. Oregon-green labeled tubulin is polymerized *in vitro*, and the resulting microtubules isolated, stabilised with taxol, and perfused into a microscope slide/glass coverslip chamber as previously described (Marlowe et al., 1998). The chamber is then washed with BRB-80 plus 10µM taxol to stabilise microtubules and remove non-polymerized tubulin. A mixture of HeLa cytosol (2mg/ml), MgATP (200µM), fluorescently-labeled endosomes (3mg/ml), purified bovine haemoglobin (3mg/ml; Sigma), and an anti-fade system (Howard and Hyman, 1993), in the presence or absence of the candidate drug, is perfused

into the chamber, and images of fluorescently-labeled microtubules (FITC filter set) or endosomes (Rhodamine filter set) are collected at 2sec-intervals using a time-lapse fluorescence videomicroscope. All image acquisition, processing, and analysis of movies is performed using the NIH Image v1.60 software package. Endosome movements are defined as linear, vectorial motions that occur on fluorescent microtubules over four or more consecutive images. These are discriminated from tethered Brownian motions, which generally display non-linear shaking, or flip randomly back and forth from image to image. Movements are counted from at least three and in most cases many more, individual movies, averaged and population significant differences calculated using the Excel spreadsheet program (Microsoft, Inc.). Substances that are able to inhibit the motility of early endosomes with microtubules below the standard fusion activity in the presence of cytosol and an ATP-regenerating system can be searched for. To control that the inhibition is targeted to a Rab5 effector (by inhibiting the Rab5-Rab5 effector interaction or the Rab5 effector activity), the inhibitory effect of the drug may be rescued by increasing concentrations of that particular Rab5 effector. For this a titration curve of the drug in the presence of increasing concentration of the Rab5 effector is necessary.

#### Assay for activity 5

This assay describes the measurement of cell motility in vitro. The assay may be standardised for high throughput screening.

20

To address the effect of drugs on cell motility, BBCE cells or other tumor cell lines are trypsinized and plated at a density of 1,500 cells per cm<sup>2</sup>. 24h later the cells are stimulated with bFGF or other growth factors and subjected to Time-Lapse Video Microscopy (TLVM). The cells for TLVM are grown on coverslips and mounted into aluminum chambers as described in (Bradke and Dotti, 1997). Briefly, aluminum is cut into 7.5cm x 2.5cm x 2mm rectangles, the dimensions are similar to a standard glass slide and fit onto the microscope stage. In the centre a circular hole is cut of 8 mm diameter, and around it 4mm area is milled from both sides. A glass coverslip is inserted onto one side of the chamber, sealed with a lubricant, medium is then added to fill the chamber, cells grown on coverslips are then inverted onto the other side of the metal slide and sealed with lubricant. TLVM is carried out using a standard video microscopy set up. A dedicated automatic program was developed to detect and track fluorescently labeled cells as they move.

30

Example 3:

A novel Procedure for the Purification of Rab5 effectors.

120l of bacterial DH5 $\alpha$  cells were grown to express GST-Rab5 for the large scale isolation of Rab5 effectors. The cells were induced, harvested, and broken according to manufacturer instructions (Pharmacia). Lysis buffer (2500ml) consisted of a PBS solution containing 100 $\mu$ M GDP, 5mM MgCl<sub>2</sub>, 5mM 2-mercaptoethanol, 5 $\mu$ g/ml DNAase, 5 $\mu$ g/ml RNase, and a cocktail of protease inhibitors. High speed supernatant of the cell lysate was incubated with 20ml glutathione sepharose beads (Pharmacia) at 4°C for 2h under rotation. Subsequently, beads were loaded on a column and washed with lysis buffer without DNAase, RNase, and protease inhibitors. This procedure resulted in 1g of GST-Rab5 bound to the column. The molecule is recovered in the GDP-bound inactive form and nucleotide exchange is necessary to convert it into the GTP-bound form to allow effector binding.

The present inventors have designed an alternative procedure that overcomes the low efficiency normally affecting this step. The modification consists in the use of aluminum fluoride (AlF<sub>3</sub>) which when bound to Ras:GDP and Mg<sup>2+</sup> has been shown to mimic the transition state for GTP-hydrolysis of the GTPase complexed to GAP. The inventors have obtained evidence, however, that the method can also be applied for the complex between Rab5:GDP:Mg<sup>2+</sup>:AlF<sub>3</sub> and its effectors. In other words, it is possible to obtain a complex consisting of Rab5, GDP, Mg<sup>2+</sup>, AlF<sub>3</sub>, and Rab5 effectors. This procedure has for example allowed the inventors to purify at large scale EEA1 and Rabankyrin-5, suggesting that this method can be applied for the identification of novel effectors and regulators for small GTPases.

Example 4:

Construction and expression of EGFP-Rab5

pEGFP-Rab5 was constructed by PCR amplification of the human Rab5a cDNA from pGEM-myc-Rab5 using the primers CCCAAGCTTATGGCTAGTCGAGGCGCAACA and AACTGCAGTTAGTTACTACAACACTGATT followed by cloning of the HindIII-PstI fragment from the PCR product into a pEGFP-C3 expression vector (Clontech, Inc.). A431 cells were grown to ~80% confluency in 10cm petri dishes and transfected with 20-30 $\mu$ g of plasmid DNA using a calcium phosphate based protocol. Stably transfected clonal lines were isolated after incubation in selective (G418; 0.5 $\mu$ g/ml) growth medium for 7-10d and checked for GFP

fluorescence on endosomal structures. Despite the presence of the selectable marker, variable levels of expression of EGFP-Rab5 in these cells were observed.

#### Time-lapse fluorescence videomicroscopy of EGFP-Rab5 *in vivo*

5 Cells were grown on glass coverslips and were transferred to custom-built aluminum microscope slide chambers (EMBL workshop, Heidelberg) just prior to observation. Unless otherwise stated, cells expressing average levels of EGFP-Rab5 were selected and analyzed on a Zeiss Axioskop microscope using 100X/1.40 plan-Apochromat lens with a temperature-controlled objective sleeve attached (EMBL workshop, Heidelberg). Time-lapse imaging was performed, collecting  
10 images at 2sec-intervals using a computer-controlled shutter (Uniblitz, Inc.) with illumination by a 100W mercury arc lamp attenuated with two heat reflection filters and a KG-1 heat absorbance filter (Zeiss). GFP-fluorescence was visualised with Hi-Q FIT, or GFP filter sets (Chroma Technologies, Inc.), and images were acquired using a COHU 4913 CCIR video camera with on-chip integration controlled by the NIH-Image v1.60 software package.

#### Example 5:

##### Microtubule spin-down assay

75 to 100µg of purified early endosomes were incubated at room temperature for 20min with 100µg HeLa cytosol protein in a reaction brought to a final volume of 50µl by addition of BRB-  
20 80 (80mM K-Pipes, 1mM MgCl<sub>2</sub>, 1mM EGTA, pH6.8). 10µl of taxol-stabilized microtubules (100µg tubulin equivalent) was added and this mix was incubated for 10min at room temperature, and then overlaid on 600µl of a 35% (w/v) sucrose cushion. After sedimentation at 100,000 x g for 20min at 22°C in a TLA 100.4 rotor, the upper layer was removed, the cushion was washed with 100µl BRB-80 and then also removed. The remaining pellet was resuspended  
25 in 30µl 2x SDS-PAGE buffer plus 30µl dH<sub>2</sub>O, and analysed by SDS-PAGE and immunoblotting.

#### Example 6:

##### *In vitro* endosome motility assays

Early endosomes, and cytosol were isolated as previously described, except HeLa spinner culture  
30 cells were allowed to internalise rhodamine-labeled transferrin for 10min at 37°C in order to label early endosomes. Oregon-green labeled tubulin was polymerized *in vitro*, and the resulting microtubules isolated, stabilised with taxol, and perfused into a microscope slide/glass coverslip chamber as previously described. Alternatively, purified centrosomes were allowed to stick to the



glass surfaces of the chamber. Microtubule asters were then polymerized *in situ* by 30min incubation of the chamber at 37°C with fluorescently-labeled tubulin (4mg/ml) and 1mM GTP in BRB-80. The chamber was then washed with BRB-80 plus 10µM taxol to stabilise microtubules and remove non-polymerized tubulin. A mixture of HeLa cytosol (2mg/ml), MgATP (200µM),  
5 fluorescently-labeled endosomes (3mg/ml), purified bovine haemoglobin (3mg/ml; Sigma), and an anti-fade system was perfused into the chamber, and images of fluorescently-labeled microtubules (FITC filter set) or endosomes (Rhodamine filter set) were collected at 2sec-intervals using the time-lapse fluorescence videomicroscope setup described above.

#### 10 Analysis and quantification of videos

All image acquisition, processing, and analysis of movies was performed using the NIH Image v1.60 software package. Endosome movements were defined as linear, vectorial motions that occurred on fluorescent microtubules over four or more consecutive images. These were discriminated from tethered Brownian motions, which generally displayed non-linear shaking, or  
15 flipped randomly back and forth from image to image. Movements were counted from at least three and in most cases many more, individual movies, averaged and population significant differences calculated using the Excel spreadsheet program (Microsoft, Inc.).

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## CLAIMS

1. Use of an effector of a GTPase as a target in an *in vitro* or *in vivo* assay to detect  
5 substances useful as pharmaceutical agents for the prophylaxis and/or treatment of cancer  
and other proliferative, invasive or cell migration disorders such as endometriosis,  
atherosclerosis, inflammatory and allergic diseases, infectious diseases, diabetes,  
Alzheimer's disease and skin repair diseases such as psoriasis.
2. Use of claim 1, wherein the GTPase is of the Rab family.
3. Use of claim 2, wherein the GTPase is any of Rab4, Rab5, Rab7, Rab11, Rab17, Rab18,  
10 and Rab22.
4. Use of any of the preceding claims, wherein the infectious disease is AIDS, tuberculosis,  
15 pseudotuberculosis, cholera, malaria, gastroenteritis, enteric fever, typhus, those diseases  
caused by pathogens (bacteria or organisms) such as Mycobacterium, Staphylococcus,  
Toxoplasma, Trypanosoma, Listeria, Salmonella, Legionella, Leishmania, Coxiella,  
Shigella, Yersinia, Neisseria, Vibrio, Bartonella, or any other infectious disease caused by  
an infectious agent that infects cells by the endocytic route and resides intracellularly in  
20 phagosomes escaping the cellular killing mechanisms.
5. Use of any of claims 1 to 3, wherein the cancer is a benign tumor, a malignant tumor, a  
carcinoma, a sarcoma, a melanoma, a leukemia, a glioma, or a neuroblastoma, in  
particular a lung carcinoma, an osteosarcoma, a lymphoma, a soft tissue sarcoma, a breast  
25 carcinoma, a bile cancer, a cervix carcinoma, a cancer of the (small) intestine, of the  
kidneys, of the cavity of the mouth, a penis carcinoma, an ovary cancer, a stomach cancer,  
a cancer of the tongue, a brain cancer, a bladder carcinoma, a prostate carcinoma, a liver  
carcinoma, a carcinoma of the pancreas, and every tumor that invades other tissues and  
organs distinct from its site of origin.
- 30 6. Use of any of the preceding claims, wherein the assay is carried out in the presence of one  
or more GTPase effector/regulator molecule(s) which is/are either native and  
biochemically purified from a vertebrate, or recombinant and biochemically purified from

bacterial cultures, from yeast cultures, or from other cultured eukaryotic cells, in either case labeled by a covalent modification or radioactivity suitable for use in the assay.

7. Use of claim 6, wherein the assay is carried out in the simultaneous presence of at least one type of GTPase and/or endosomal membrane fractions fluorescently labeled or labeled by any other modification that allows its detection, and/or cytosolic extracts, and/or an ATP-regenerating system and/or a number of chemicals to be tested for their suitability as an anti-cancer or anti-infectious diseases drug.

8. Use of any of the preceding claims, wherein the substance useful as pharmaceutical agent is a molecule/substance that carries one or more of the following functional groups: a halide atom bound to an alkyl, alkenyl, alkynyl or aryl residue, an alcohol group (primary, secondary, tertiary), an ether group, a carbonyl function (aldehyde or ketone), a carboxylic acid group, a carboxylic anhydride group, a carbamoyl group, a haloformyl group, a cyano group, an ester group including a lactone group, a benzyl, phenyl, tolyl, tosyl, sulfonyl group, an amino group (primary, secondary, tertiary), an isocyanate, a cyanate, a thioisocyanate, a thiocyanate, a carbamate, an azide, a diazo group, and a quinone group; or is an organometallic compound, a sterol moiety(ies)-containing molecule, a  $\beta$ -hydroxy carboxylic acid, an inorganic acid or complex such as a metallocene, a nucleic acid, a cytokine, a hormone, an antibody, or an oligopeptide comprising up to 20, preferably 8, 10, or 12, amino acid residues.

9. Use of claim 8, wherein the antibody is a polyclonal or monoclonal antibody, or a fragment thereof, humanised or human, inhibitory or stimulatory, raised against and targeted towards any of the aforementioned GTPase effectors.

10. Use of claim 8, wherein the nucleic acid is genomic DNA, cDNA, or mRNA, or a fragment there, an oligonucleotide, an oligoribonucleotide, all being based on or derived from any of the GTPase effector having any of the sequences as depicted in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, and 15, or gene therapy vectors derived from the aforementioned GTPase effector gene sequences.

11. A kit useful for carrying out the assay of any of the preceding claims, the kit comprising in a suitable means at least one GTPase effector/regulator molecule which is either native and biochemically purified from a vertebrate, or recombinant and biochemically purified from bacterial cultures, from yeast cultures, or from other cultured eukaryotic cells, in  
5 either case labeled by a covalent modification or by radioactivity suitable for the use in the assay.
12. The kit of claim 11, wherein the kit further comprises at least one type of GTPase and/or endosomal membrane fractions fluorescently labeled or labeled by any other modification  
10 that allows its detection, and/or cytosolic extracts, and/or an ATP-regenerating system and/or a number of chemicals to be tested for their suitability as a drug effective against any of the diseases depicted in claims 1, 4, and 5.
13. The kit of claim 12, wherein the at least one GTPase is/are one or more of the GTPases  
15 Rab4, Rab5, Rab7, Rab11, Rab17, Rab18, and Rab22.

## SEQUENCE LISTING

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5 <120> A new Assay to Detect Substances Useful for the Therapy  
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	ttcaactacc	aggtcgccac	caagcagctc	ctgttccgac	tgctggatat	gctgtccaag	3300
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	actcgcaaac	accactgtcg	tcactgcgga	cgtcttcttt	gccataaatg	ctcgaccaag	3420
	gagattccta	ttataaagtt	tgatctgaac	aagcctgtgc	gggtttgcaa	catttgtttt	3480
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&lt;210&gt; 2

&lt;211&gt; 1169

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 2

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    1             5             10             15

    Arg Gln Glu Tyr Val Lys Leu Gln Lys Lys Leu Ala Glu Thr Glu Lys
              20             25             30
10

    Arg Cys Ala Leu Leu Ala Ala Gln Ala Asn Lys Glu Ser Ser Ser Glu
      35             40             45

    Ser Phe Ile Ser Arg Leu Leu Ala Ile Val Ala Asp Leu Tyr Glu Gln
15      50             55             60

    Glu Gln Tyr Ser Asp Leu Lys Ile Lys Val Gly Asp Arg His Ile Ser
    65             70             75             80

20  Ala His Lys Phe Val Leu Ala Ala Arg Ser Asp Ser Trp Ser Leu Ala
      85             90             95

    Asn Leu Ser Ser Thr Lys Glu Leu Asp Leu Ser Asp Ala Asn Pro Glu
      100            105            110
25

    Val Thr Met Thr Met Leu Arg Trp Ile Tyr Thr Asp Glu Leu Glu Phe
      115            120            125

    Arg Glu Asp Asp Val Phe Leu Thr Glu Leu Met Lys Leu Ala Asn Arg
30      130            135            140

    Phe Gln Leu Gln Leu Leu Arg Glu Arg Cys Glu Lys Gly Val Met Ser
    145            150            155            160

35  Leu Val Asn Val Arg Asn Cys Ile Arg Phe Tyr Gln Thr Ala Glu Glu
      165            170            175

    Leu Asn Ala Ser Thr Leu Met Asn Tyr Cys Ala Glu Ile Ile Ala Ser
      180            185            190
40

    His Trp Asp Asp Leu Arg Lys Glu Asp Phe Ser Ser Met Ser Ala Gln
      195            200            205

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Leu Leu Tyr Lys Met Ile Lys Ser Lys Thr Glu Tyr Pro Leu His Lys  
 210 215 220

Ala Ile Lys Val Glu Arg Glu Asp Val Val Phe Leu Tyr Leu Ile Glu  
 5 225 230 235 240

Met Asp Ser Gln Leu Pro Gly Lys Leu Asn Glu Ala Asp His Asn Gly  
 245 250 255

10 Asp Leu Ala Leu Asp Leu Ala Leu Ser Arg Arg Leu Glu Ser Ile Ala  
 260 265 270

Thr Thr Leu Val Ser His Lys Ala Asp Val Asp Met Val Asp Lys Ser  
 275 280 285

15 Gly Trp Ser Leu Leu His Lys Gly Ile Gln Arg Gly Asp Leu Phe Ala  
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Ala Thr Phe Leu Ile Lys Asn Gly Ala Phe Val Asn Ala Ala Thr Leu  
 20 305 310 315 320

Gly Ala Gln Glu Thr Pro Leu His Leu Val Ala Leu Tyr Ser Ser Lys  
 325 330 335

25 Lys His Ser Ala Asp Val Met Ser Glu Met Ala Gln Ile Ala Glu Ala  
 340 345 350

Leu Leu Gln Ala Gly Ala Asn Pro Asn Met Gln Asp Ser Lys Gly Arg  
 355 360 365

30 Thr Pro Leu His Val Ser Ile Met Ala Gly Asn Glu Tyr Val Phe Ser  
 370 375 380

Gln Leu Leu Gln Cys Lys Gln Leu Asp Leu Glu Leu Lys Asp His Glu  
 35 385 390 395 400

Gly Ser Thr Ala Leu Trp Leu Ala Val Gln His Ile Thr Val Ser Ser  
 405 410 415

40 Asp Gln Ser Val Asn Pro Phe Glu Asp Val Pro Val Val Asn Gly Thr  
 420 425 430

Ser Phe Asp Glu Asn Ser Phe Ala Ala Arg Leu Ile Gln Arg Gly Ser  
 435 440 445

	His Thr Asp Ala Pro Asp Thr Ala Thr Gly Asn Cys Leu Leu Gln Arg	
	450	455 460
	Ala Ala Gly Ala Gly Asn Glu Ala Ala Ala Leu Phe Leu Ala Thr Asn	
5	465	470 475 480
	Gly Ala His Val Asn His Arg Asn Lys Trp Gly Glu Thr Pro Leu His	
		485 490 495
10	Thr Ala Cys Arg His Gly Leu Ala Asn Leu Thr Ala Glu Leu Leu Gln	
		500 505 510
	Gln Gly Ala Asn Pro Asn Leu Gln Thr Glu Glu Ala Leu Pro Leu Pro	
		515 520 525
15	Lys Glu Ala Ala Ser Leu Thr Ser Leu Ala Asp Ser Val His Leu Gln	
		530 535 540
	Thr Pro Leu His Met Ala Ile Ala Tyr Asn His Pro Asp Val Val Ser	
20	545	550 555 560
	Val Ile Leu Glu Gln Lys Ala Asn Ala Leu His Ala Thr Asn Asn Leu	
		565 570 575
25	Gln Ile Ile Pro Asp Phe Ser Leu Lys Asp Ser Arg Asp Gln Thr Val	
		580 585 590
	Leu Gly Leu Ala Leu Trp Thr Gly Met His Thr Ile Ala Ala Gln Leu	
		595 600 605
30	Leu Gly Ser Gly Ala Ala Ile Asn Asp Thr Met Ser Asp Gly Gln Thr	
		610 615 620
	Leu Leu His Met Ala Ile Gln Arg Gln Asp Ser Lys Ser Ala Leu Phe	
35	625	630 635 640
	Leu Leu Glu His Gln Ala Asp Ile Asn Val Arg Thr Gln Asp Gly Glu	
		645 650 655
40	Thr Ala Leu Gln Leu Ala Ile Arg Asn Gln Leu Pro Leu Val Val Asp	
		660 665 670
	Ala Ile Cys Thr Arg Gly Ala Asp Met Ser Val Pro Asp Glu Lys Gly	
		675 680 685

Asn Pro Pro Leu Trp Leu Ala Leu Ala Asn Asn Leu Glu Asp Ile Ala  
 690 695 700

5 Ser Thr Leu Val Arg His Gly Cys Asp Ala Thr Cys Trp Gly Pro Gly  
 705 710 715 720

Pro Gly Gly Cys Leu Gln Thr Leu Leu His Arg Ala Ile Asp Glu Asn  
 725 730 735

10 Asn Glu Pro Thr Ala Cys Phe Leu Ile Arg Ser Gly Cys Asp Val Asn  
 740 745 750

Ser Pro Arg Gln Pro Gly Ala Asn Gly Glu Gly Glu Glu Glu Ala Arg  
 755 760 765

15 Asp Gly Gln Thr Pro Leu His Leu Ala Ala Ser Trp Gly Leu Glu Glu  
 770 775 780

20 Thr Val Gln Cys Leu Leu Glu Phe Gly Ala Asn Val Asn Ala Gln Asp  
 785 790 795 800

Ala Glu Gly Arg Thr Pro Ile His Val Ala Ile Ser Ser Gln His Gly  
 805 810 815

25 Val Ile Ile Gln Leu Leu Val Ser His Pro Asp Ile His Leu Asn Val  
 820 825 830

Arg Asp Arg Gln Gly Leu Thr Pro Phe Ala Cys Ala Met Thr Phe Lys  
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30 Asn Asn Lys Ser Ala Glu Ala Ile Leu Lys Arg Glu Ser Gly Ala Ala  
 850 855 860

35 Glu Gln Val Asp Asn Lys Gly Arg Asn Phe Leu His Val Ala Val Gln  
 865 870 875 880

Asn Ser Asp Ile Glu Ser Val Leu Phe Leu Ile Ser Val His Ala Asn  
 885 890 895

40 Val Asn Ser Arg Val Gln Asp Ala Ser Lys Leu Thr Pro Leu His Leu  
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Ala Val Gln Ala Gly Ser Glu Ile Ile Val Arg Asn Leu Leu Leu Ala  
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Gly Ala Lys Val Asn Glu Leu Thr Lys His Arg Gln Thr Ala Leu His  
 930 935 940

Leu Ala Ala Gln Gln Asp Leu Pro Thr Ile Cys Ser Val Leu Leu Glu  
 5 945 950 955 960

Asn Gly Val Asp Phe Ala Ala Val Asp Glu Asn Gly Asn Asn Ala Leu  
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10 His Leu Ala Val Met His Gly Arg Leu Asn Asn Ile Arg Val Leu Leu  
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Thr Glu Cys Thr Val Asp Ala Glu Ala Phe Asn Leu Arg Gly Gln Ser  
 995 1000 1005

15 Pro Leu His Ile Leu Gly Gln Tyr Gly Lys Glu Asn Ala Ala Ala Ile  
 1010 1015 1020

Phe Asp Leu Phe Leu Glu Cys Met Pro Gly Tyr Pro Leu Asp Lys Pro  
 20 1025 1030 1035 1040

Asp Ala Asp Gly Ser Thr Val Leu Leu Leu Ala Tyr Met Lys Gly Asn  
 1045 1050 1055

25 Ala Asn Leu Cys Arg Ala Ile Val Arg Ser Gly Ala Arg Leu Gly Val  
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Asn Asn Asn Gln Gly Val Asn Ile Phe Asn Tyr Gln Val Ala Thr Lys  
 1075 1080 1085

30 Gln Leu Leu Phe Arg Leu Leu Asp Met Leu Ser Lys Glu Pro Pro Trp  
 1090 1095 1100

Cys Asp Gly Ser Tyr Cys Tyr Glu Cys Thr Ala Arg Phe Gly Val Thr  
 35 1105 1110 1115 1120

Thr Arg Lys His His Cys Arg His Cys Gly Arg Leu Leu Cys His Lys  
 1125 1130 1135

40 Cys Ser Thr Lys Glu Ile Pro Ile Ile Lys Phe Asp Leu Asn Lys Pro  
 1140 1145 1150

Val Arg Val Cys Asn Ile Cys Phe Asp Val Leu Thr Leu Gly Gly Val  
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Ser

&lt;210&gt; 3

5 &lt;211&gt; 3390

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;400&gt; 3

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25 <210> 4
    <211> 1129
    <212> PRT
    <213> Homo sapiens

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Arg Gln Glu Tyr Val Lys Leu Gln Lys Lys Leu Ala Glu Thr Glu Lys
35              20              25              30

Arg Cys Ala Leu Leu Ala Ala Gln Ala Asn Lys Glu Ser Ser Ser Glu
    35              40              45

40 Ser Phe Ile Ser Arg Leu Leu Ala Ile Val Ala Asp Leu Tyr Glu Gln
    50              55              60

Glu Gln Tyr Ser Ala Asn Pro Glu Val Thr Met Thr Met Leu Arg Trp
    65              70              75              80

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	Ile	Tyr	Thr	Asp	Glu	Leu	Glu	Phe	Arg	Glu	Asp	Asp	Val	Phe	Leu	Thr	
					85					90					95		
5	Glu	Leu	Met	Lys	Leu	Ala	Asn	Arg	Phe	Gln	Leu	Gln	Leu	Leu	Arg	Glu	
				100					105					110			
	Arg	Cys	Glu	Lys	Gly	Val	Met	Ser	Leu	Val	Asn	Val	Arg	Asn	Cys	Ile	
			115					120					125				
10	Arg	Phe	Tyr	Gln	Thr	Ala	Glu	Glu	Leu	Asn	Ala	Ser	Thr	Leu	Met	Asn	
		130					135					140					
	Tyr	Cys	Ala	Glu	Ile	Ile	Ala	Ser	His	Trp	Asp	Asp	Leu	Arg	Lys	Glu	
	145					150				155					160		
15	Asp	Phe	Ser	Ser	Met	Ser	Ala	Gln	Leu	Leu	Tyr	Lys	Met	Ile	Lys	Ser	
					165					170					175		
	Lys	Thr	Glu	Tyr	Pro	Leu	His	Lys	Ala	Ile	Lys	Val	Glu	Arg	Glu	Asp	
20			180						185					190			
	Val	Val	Phe	Leu	Tyr	Leu	Ile	Glu	Met	Asp	Ser	Gln	Leu	Pro	Gly	Lys	
			195					200					205				
25	Leu	Asn	Glu	Ala	Asp	His	Asn	Gly	Asp	Leu	Ala	Leu	Asp	Leu	Ala	Leu	
		210					215					220					
	Ser	Arg	Arg	Leu	Glu	Ser	Ile	Ala	Thr	Thr	Leu	Val	Ser	His	Lys	Ala	
	225					230				235					240		
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				245					250					255			
	Ile	Gln	Arg	Gly	Asp	Leu	Phe	Ala	Ala	Thr	Phe	Leu	Ile	Lys	Asn	Gly	
35			260					265					270				
	Ala	Phe	Val	Asn	Ala	Ala	Thr	Leu	Gly	Ala	Gln	Glu	Thr	Pro	Leu	His	
			275					280					285				
40	Leu	Val	Ala	Leu	Tyr	Ser	Ser	Lys	Lys	His	Ser	Ala	Asp	Val	Met	Ser	
		290					295					300					
	Glu	Met	Ala	Gln	Ile	Ala	Glu	Ala	Leu	Leu	Gln	Ala	Gly	Ala	Asn	Pro	
	305					310					315				320		

	Asn Met Gln Asp Ser Lys Gly Arg Thr Pro Leu His Val Ser Ile Met	
	325	330 335
5	Ala Gly Asn Glu Tyr Val Phe Ser Gln Leu Leu Gln Cys Lys Gln Leu	
	340	345 350
	Asp Leu Glu Leu Lys Asp His Glu Gly Ser Thr Ala Leu Trp Leu Ala	
	355	360 365
10	Val Gln His Ile Thr Val Ser Ser Asp Gln Ser Val Asn Pro Phe Glu	
	370	375 380
	Asp Val Pro Val Val Asn Gly Thr Ser Phe Asp Glu Asn Ser Phe Ala	
	385	390 395 400
15	Ala Arg Leu Ile Gln Arg Gly Ser His Thr Asp Ala Pro Asp Thr Ala	
	405	410 415
	Thr Gly Asn Cys Leu Leu Gln Arg Ala Ala Gly Ala Gly Asn Glu Ala	
20	420	425 430
	Ala Ala Leu Phe Leu Ala Thr Asn Gly Ala His Val Asn His Arg Asn	
	435	440 445
25	Lys Trp Gly Glu Thr Pro Leu His Thr Ala Cys Arg His Gly Leu Ala	
	450	455 460
	Asn Leu Thr Ala Glu Leu Leu Gln Gln Gly Ala Asn Pro Asn Leu Gln	
	465	470 475 480
30	Thr Glu Glu Ala Leu Pro Leu Pro Lys Glu Ala Ala Ser Leu Thr Ser	
	485	490 495
	Leu Ala Asp Ser Val His Leu Gln Thr Pro Leu His Met Ala Ile Ala	
35	500	505 510
	Tyr Asn His Pro Asp Val Val Ser Val Ile Leu Glu Gln Lys Ala Asn	
	515	520 525
40	Ala Leu His Ala Thr Asn Asn Leu Gln Ile Ile Pro Asp Phe Ser Leu	
	530	535 540
	Lys Asp Ser Arg Asp Gln Thr Val Leu Gly Leu Ala Leu Trp Thr Gly	
	545	550 555 560

## 12

	Met	His	Thr	Ile	Ala	Ala	Gln	Leu	Leu	Gly	Ser	Gly	Ala	Ala	Ile	Asn	
					565					570					575		
	Asp	Thr	Met	Ser	Asp	Gly	Gln	Thr	Leu	Leu	His	Met	Ala	Ile	Gln	Arg	
5				580					585						590		
	Gln	Asp	Ser	Lys	Ser	Ala	Leu	Phe	Leu	Leu	Glu	His	Gln	Ala	Asp	Ile	
				595				600						605			
10	Asn	Val	Arg	Thr	Gln	Asp	Gly	Glu	Thr	Ala	Leu	Gln	Leu	Ala	Ile	Arg	
		610					615					620					
	Asn	Gln	Leu	Pro	Leu	Val	Val	Asp	Ala	Ile	Cys	Thr	Arg	Gly	Ala	Asp	
	625					630					635				640		
15	Met	Ser	Val	Pro	Asp	Glu	Lys	Gly	Asn	Pro	Pro	Leu	Trp	Leu	Ala	Leu	
					645					650					655		
	Ala	Asn	Asn	Leu	Glu	Asp	Ile	Ala	Ser	Thr	Leu	Val	Arg	His	Gly	Cys	
20				660					665					670			
	Asp	Ala	Thr	Cys	Trp	Gly	Pro	Gly	Pro	Gly	Gly	Cys	Leu	Gln	Thr	Leu	
			675					680					685				
25	Leu	His	Arg	Ala	Ile	Asp	Glu	Asn	Asn	Glu	Pro	Thr	Ala	Cys	Phe	Leu	
		690					695					700					
	Ile	Arg	Ser	Gly	Cys	Asp	Val	Asn	Ser	Pro	Arg	Gln	Pro	Gly	Ala	Asn	
	705					710					715				720		
30	Gly	Glu	Gly	Glu	Glu	Glu	Ala	Arg	Asp	Gly	Gln	Thr	Pro	Leu	His	Leu	
					725					730					735		
	Ala	Ala	Ser	Trp	Gly	Leu	Glu	Glu	Thr	Val	Gln	Cys	Leu	Leu	Glu	Phe	
35				740					745					750			
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			755					760					765				
40	Val	Ala	Ile	Ser	Ser	Gln	His	Gly	Val	Ile	Ile	Gln	Leu	Leu	Val	Ser	
		770					775					780					
	His	Pro	Asp	Ile	His	Leu	Asn	Val	Arg	Asp	Arg	Gln	Gly	Leu	Thr	Pro	
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	Phe	Ala	Cys	Ala	Met	Thr	Phe	Lys	Asn	Asn	Lys	Ser	Ala	Glu	Ala	Ile	
					805					810					815		
	Leu	Lys	Arg	Glu	Ser	Gly	Ala	Ala	Glu	Gln	Val	Asp	Asn	Lys	Gly	Arg	
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	Asn	Phe	Leu	His	Val	Ala	Val	Gln	Asn	Ser	Asp	Ile	Glu	Ser	Val	Leu	
				835				840					845				
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	Lys	His	Arg	Gln	Thr	Ala	Leu	His	Leu	Ala	Ala	Gln	Gln	Asp	Leu	Pro	
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	Arg	Ser	Gly	Ala	Arg	Leu	Gly	Val	Asn	Asn	Asn	Gln	Gly	Val	Asn	Ile	
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Phe Asn Tyr Gln Val Ala Thr Lys Gln Leu Leu Phe Arg Leu Leu Asp  
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Cys Thr Ala Arg Phe Gly Val Thr Thr Arg Lys His His Cys Arg His  
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10 Cys Gly Arg Leu Leu Cys His Lys Cys Ser Thr Lys Glu Ile Pro Ile  
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 Tyr Glu Glu Glu His Ser Gly Glu Asp Arg Asp Val Lys Gly Gln Ile  
 35 40 45  
 40 Lys Ser Leu Val Gln Lys Ala Lys Lys Ala Lys Asp Arg Leu Leu Lys  
 50 55 60  
 Arg Glu Gly Asp Asp Arg Ala Glu Ser Gly Thr Gln Gly Tyr Glu Ser  
 65 70 75 80

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	Leu	Ile	Ser	Leu	Pro	Leu	Ala	Asn	Lys	Leu	Thr	Ser	Ala	Ser	Lys	Glu	
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	Gly	Ser	Arg	Arg	Gly	Ser	Ile	Ser	Ser	Met	Ser	Ser	Val	Ser	Ser	Val	
	225					230				235						240	
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	Asp	Thr	Leu	Leu	Lys	Arg	Glu	Gln	Gln	Ile	Asp	Glu	Lys	Glu	His	Thr	
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	Pro	Asp	Ile	Val	Lys	Leu	Tyr	Glu	Lys	Leu	Arg	Leu	Cys	Met	Glu	Lys	
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40	Val	Asp	Gln	Lys	Ala	Pro	Glu	Tyr	Ile	Arg	Met	Ala	Ala	Ser	Leu	Asn	
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	Ala	Gly	Glu	Thr	Thr	Tyr	Ser	Leu	Glu	His	Ala	Ser	Asp	Leu	Arg	Val	
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Arg Leu Gln Arg Met Ile Arg Tyr Ser Ala Thr Leu Phe Val Gln Glu  
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10 Lys Leu Leu Gly Leu Met Ser Leu Pro Thr Lys Glu Gln Phe Glu Glu  
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15 Arg Gln Ala Ala Leu Glu Ser Gln Arg Arg Leu Glu Glu Arg Gln Ser  
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Gly Leu Ala Ser Arg Ala Ala Asn Gly Glu Val Ala Ser Leu Arg Arg  
 20 420 425 430

Gly Pro Ala Pro Leu Arg Lys Ala Glu Gly Trp Leu Pro Leu Ser Gly  
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25 Gly Gln Gly Gln Ser Glu Asp Ser Asp Pro Leu Leu Gln Gln Ile His  
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Asn Ile Thr Ser Phe Ile Arg Gln Ala Lys Ala Ala Gly Arg Met Asp  
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30 Glu Val Arg Thr Leu Gln Glu Xaa Leu Arg Gln Leu Gln Asp Glu Tyr  
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Asp Gln Gln Gln Thr Glu Lys Ala Ile Glu Leu Ser Arg Arg Gln Ala  
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Glu Glu Glu Asp Leu Gln Arg Glu Gln Leu Gln Met Leu Arg Glu Arg  
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40 Glu Leu Glu Arg Glu Arg Glu Gln Phe Arg Val Ala Ser Leu His Thr  
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Arg Thr Arg Ser Leu Asp Phe Arg Glu Ile Gly Pro Phe Gln Leu Glu  
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	Pro Ser Arg Glu Pro Arg Thr His Leu Ala Tyr Ala Leu Asp Leu Gly	
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	Ser Thr Gln Pro Thr Arg Val Trp Ser Gly Pro Pro Ala Val Gly Gln	
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10	Glu Arg Leu Pro Gln Ser Ser Met Pro Gln Gln His Glu Gly Pro Ser	
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	Leu Asn Pro Phe Asp Glu Glu Asp Leu Ser Ser Pro Met Glu Glu Ala	
	625	630 635 640
15	Thr Thr Gly Pro Pro Ala Ala Gly Val Ser Leu Asp Pro Ser Ala Arg	
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	Ile Leu Lys Glu Tyr Asn Pro Phe Glu Glu Glu Asp Glu Glu Glu Glu	
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	Ala Val Ala Gly Asn Pro Phe Ile Gln Pro Asp Ser Pro Ala Pro Asn	
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25	Pro Phe Ser Glu Glu Asp Glu His Pro Gln Gln Arg Leu Ser Ser Pro	
	690	695 700
	Leu Val Pro Gly Asn Pro Phe Glu Glu Pro Thr Cys Ile Asn Pro Phe	
	705	710 715 720
30	Glu Met Asp Ser Asp Ser Gly Pro Glu Ala Glu Glu Pro Ile Glu Glu	
	725	730 735
	Glu Leu Leu Leu Gln Gln Ile Asp Asn Ile Lys Ala Tyr Ile Phe Asp	
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	Ala Lys Gln Cys Gly Arg Leu Asp Glu Val Glu Val Leu Thr Glu Asn	
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5      Lys Phe Pro Val Gln Glu Asn Ala Ser His Leu His Gly Arg Leu Met  
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Leu Leu His Ser Leu Pro Cys Phe Ile Glu Lys Asp Leu Lys Glu Ala  
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10    Leu Thr Gln Phe Ile Glu Glu Glu Ser Leu Ser Asp Tyr Asp Arg Asp  
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Ala Glu Ala Ser Leu Ala Ala Val Lys Ser Gly Glu Val Asp Leu His  
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15    Gln Leu Ala Ser Thr Trp Ala Lys Ala Tyr Ala Glu Thr Thr Leu Glu  
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His Ala Arg Pro Glu Glu Pro Ser Trp Asp Glu Asp Phe Ala Asp Val  
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Tyr His Asp Leu Ile His Ser Pro Ala Ser Glu Thr Leu Leu Asn Leu  
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25    Glu His Asn Tyr Phe Val Ser Ile Ser Glu Leu Ile Gly Glu Arg Asp  
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Val Glu Leu Lys Lys Leu Arg Glu Arg Gln Gly Ile Glu Met Glu Lys  
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30    Val Met Gln Glu Leu Gly Lys Ser Leu Thr Asp Gln Asp Val Asn Ser  
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Leu Ala Ala Gln His Phe Glu Ser Gln Gln Asp Leu Glu Asn Lys Trp  
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Ser Asn Glu Leu Lys Gln Ser Thr Ala Ile Gln Lys Gln Glu Tyr Gln  
           210                                  215                                  220

40    Glu Trp Val Ile Lys Leu His Gln Asp Leu Lys Asn Pro Asn Asn Ser  
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Ser Leu Ser Glu Glu Ile Lys Val Gln Pro Ser Gln Phe Arg Glu Ser  
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	Lys	Asn	Ala	Glu	Arg	Asn	Tyr	Leu	Asn	Val	Leu	Pro	Gly	Glu	Phe	Tyr	
					405					410					415		
30	Ile	Thr	Arg	His	Ser	Asn	Leu	Ser	Glu	Ile	His	Val	Ala	Phe	His	Leu	
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	Ala	Ile	Met	Gly	Leu	Arg	Asn	Ile	Leu	Lys	Val	Cys	Cys	Thr	His	Asp	
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Cys Val Lys Gly Phe Met Met Glu Met Ala Ser Trp Asp Gly Gly Ile  
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Ser Arg Thr Val Gln Phe Leu Val Pro Gln Ser Ile Ser Glu Glu Met  
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Phe Tyr Gln Leu Ser Asn Met Leu Pro Gln Ile Phe Arg Val Ser Ser  
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Val Lys Leu Gln Val Lys Ala Cys Ala Leu Ser Gln Ile Asn Thr Lys  
 35 40 45

10 Leu Leu Ala Glu Met Lys Met Lys Lys Asp Leu Phe Pro Val Gly Arg  
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Glu Ile Ala Gly Ile Val Leu Asp Val Gly Ser Lys Val Ser Phe Phe  
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Gln Pro Asp Asp Glu Val Val Gly Ile Leu Pro Leu Asp Ser Glu Asp  
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20 Pro Gly Leu Cys Glu Val Val Arg Val His Glu His Tyr Leu Val His  
 100 105 110

Lys Pro Glu Lys Val Thr Trp Thr Glu Ala Ala Gly Ser Ile Arg Asp  
 115 120 125

25 Gly Val Arg Ala Tyr Thr Ala Leu His Tyr Leu Ser His Leu Ser Pro  
 130 135 140

Gly Lys Ser Val Leu Ile Met Asp Gly Ala Ser Ala Phe Gly Thr Ile  
 30 145 150 155 160

Ala Ile Gln Leu Ala His His Arg Gly Ala Lys Val Ile Ser Thr Ala  
 165 170 175

35 Cys Ser Leu Glu Asp Lys Gln Cys Leu Glu Arg Phe Arg Pro Pro Ile  
 180 185 190

Ala Arg Val Ile Asp Val Ser Asn Gly Lys Val His Val Ala Glu Ser  
 195 200 205

40 Cys Leu Glu Glu Thr Gly Gly Leu Gly Val Asp Ile Val Leu Asp Ala  
 210 215 220

Gly Val Arg Leu Tyr Ser Lys Asp Asp Glu Pro Ala Val Lys Leu Gln  
 225 230 235 240  
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/09130

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q G01N C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 42839 A (INCYTE PHARMA INC ;GUEGLER KARL J (US); HILLMAN JENNIFER L (US)) 1 October 1998 (1998-10-01) the whole document ---	1-13
X	WO 98 18942 A (INCYTE PHARMA INC ;AU YOUNG JANICE (US); BANDMAN OLGA (US)) 7 May 1998 (1998-05-07) the whole document ---	1-13
X	WO 97 00955 A (INCYTE PHARMA INC) 9 January 1997 (1997-01-09) the whole document ---	1-13
X	EP 0 856 583 A (WADA MANABU ;JAPAN SCIENCE AND TECHNOLOGY C (JP)) 5 August 1998 (1998-08-05) the whole document ---	1-13
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

20 November 2000

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/09130

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CHRISTOFORIDIS S. ET AL.: "The Rab5 effector EEA1 is a core component of endosome docking"</p> <p>NATURE, vol. 397, 18 February 1999 (1999-02-18), pages 621-625, XP002153257 cited in the application the whole document</p> <p>-----</p>	1-13

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 00/09130

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9842839 A	01-10-1998	US 5843717 A AU 6774198 A US 6010859 A	01-12-1998 20-10-1998 04-01-2000
WO 9818942 A	07-05-1998	US 6124116 A AU 4903397 A	26-09-2000 22-05-1998
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EP 0856583 A	05-08-1998	JP 10210971 A	11-08-1998